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Exercise in developing rats promotes plasticity in the prefrontal cortex: behavioral and neurobiological indications

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EXERCISE IN DEVELOPING RATS PROMOTES PLASTICITY IN THE PREFRONTAL CORTEX: BEHAVIORAL AND NEUROBIOLOGICAL INDICATIONS

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ABSTRACT

Physical exercise has repeatedly been shown to trigger positive effects on brain function including improved learning, memory, and executive functions. In addition, corresponding physiological changes have been observed, such as increased neurotrophic factors, changes in neurotransmitter concentrations, and increased dendritic spines. However, these changes have not been well described outside of the hippocampus, including the medial prefrontal cortex (mPFC), and have not been directly compared at different points of development. Because the prefrontal cortex is one of the last brain areas to fully mature, considering the age at which intervention, such as exercise, takes place is particularly important. Additionally, in human studies the data suggest that exercise has the most profound effects on prefrontal-mediated cognitive functions, while there is considerably less evidence on how exercise affects these functions in animals. The experiments presented here draw upon several well-established methodologies to explore the behavioral and physiological changes due to exercise that take place during adulthood compared to adolescence, as well as the role of mPFC sub regions in instrumental extinction and renewal.

To that end, these experiments employ conditioning paradigms using appetitive lever-pressing to assess renewal of extinguished instrumental responding following exercise or pharmacological manipulations. Additionally, because there are multiple reports suggesting that early experiences can affect prefrontal neuronal morphology, dendritic length, complexity, and spine density was examined in young or adult male rats that had access to a locked (no exercise) or unlocked (exercise) running wheel for two weeks. Furthermore, norepinephrine transporter (NET) protein expression in the mPFC was examined by Western blot. Collectively, these experiments suggest that exercise in developing, but not adult rats, reduces the expression of instrumental renewal. The precise role of the mPFC and its sub-regions (i.e., prelimbic (PL) and infralimbic (IL)) in instrumental renewal was examined, providing evidence that the behavioral consequences of physical exercise may be due to modifications not only restricted to the mPFC, but also that exercise may have preferential effects on sub-regions, or change the balance of activation. The finding that when juvenile rats exercised they showed less ABA renewal than non-exercisers, paired with the reduction of ABA renewal when the PL was inactivated (and indeed, almost an identical reduction in the two experiments) points to the deduction that exercise is affecting the PL, perhaps more so than the IL or other mPFC regions.

DEDICATION

To Drina Vurbić.

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COMPREHENSIVE LITERATURE REVIEW

Physical Exercise and the Brain

For nearly two decades, research on the neurobiological effects of exercise has consistently shown that humans and laboratory rodents experience numerous cognitive benefits from physical activity. While these benefits are wide ranging both in terms of brain areas affected and mechanisms, some of the most reliably shown changes take place in the rodent hippocampus in the form of increased neurogenesis, cell survival, and improved performance on hippocampus-reliant tasks, such as spatial learning and contextual fear conditioning (van Praag et al., 1999a, Anderson et al., 2000, Vaynman et al., 2003, Baruch et al., 2004, Vaynman et al., 2004, Hopkins and Bucci, 2010). In humans, on the other hand, exercise-induced improvements are generally seen in "higher order" cognitive abilities along with reduction of normal age-related decline, improvements following damage, and mitigation of some diseases/disorders, including Alzheimer's and Parkinson's disease, and related cognitive decline (Ahlskog, 2011, Abe, 2012, Norton et al., 2014). For example, improvements in executive function (which encompasses working memory, planning, decision making, and cognitive flexibility), which is largely mediated by the prefrontal cortex (PFC) (Funahashi, 2001) have been observed following acute and chronic physical exercise (Kramer et al., 1999, Colcombe et al., 2006, Hillman et al., 2006).

It is now well-established that rodents given the opportunity to exercise show improvements in hippocampus-dependent spatial tasks such as the Morris water maze (Fordyce and Wehner, 1993, van Praag et al., 2005), radial arm maze (Anderson et al., 2000) as well as Pavlovian fear conditioning expression (Baruch et al., 2004). Exercise-induced increases in brain derived neurotrophic factor (BDNF), neurogenesis, and cell survival in the hippocampus, particularly in CA1 and CA3 regions as well as the dentate gyrus have been associated with these behavioral changes (Neeper et al., 1996, van Praag et al., 1999b, Vaynman et al., 2004, van Praag et al., 2005). BDNF appears to be crucially involved in exercise-induced plasticity, as its downstream signaling targets, including MAPK, synapsin I, and CREB, are all implicated in synaptic plasticity (Vaynman et al., 2004). Indeed, by preventing BDNF from binding to its receptor, tyrosine kinase B (TrkB) throughout exercise intervention, Vaynman et al. (2004) demonstrated BDNF is necessary for exercise-related improvements in Morris water maze. Blocking BDNF binding (and thus its downstream effects) in the hippocampus had no effect on performance of the task in sedentary control animals (Vaynman et al., 2004). Increased BDNF levels appear to be the mechanism that mediates the exercise-related neuroplasticity and improvements in not only spatial tasks (Vaynman et al., 2004) but also consolidation of memory in fear conditioning (Schulz-Klaus et al., 2013) and novel object recognition (Fahey et al., 2008), which rely, at least partly, upon the perirhinal cortex.

Examination of the current literature on exercise's effects on the brain reveals several gaps. Chief among these is the incongruity between the focus of human and

animal research in terms of brain substrates and behaviors. Much of the work investigating exercise-related changes in rodents has focused on the hippocampus and hippocampus-dependent spatial (e.g., radial arm maze) and contextual memory (e.g., fear conditioning), while in humans the most substantial changes following exercise have not been spatial or contextual learning and memory, but rather non-hippocampus dependent functions such as executive function, including working memory, cognitive flexibility, planning, and behavioral inhibition (Hillman et al., 2003, Colcombe et al., 2004, Szabo et al., 2013) though there is evidence that exercise also positively affects the hippocampus in humans (Erickson et al., 2009, Erickson et al., 2013). For example, Colcombe et al. (2004) showed that older adults (average age approximately 66-67 years old) who had higher levels of cardiovascular fitness performed better on a flanker task, which requires the participant to respond differently to patterns of congruent versus incongruent arrows, necessitating cognitive flexibility for accurate performance. Difference in reaction time between presentations of congruent and incongruent arrows was measured, as were patterns of brain activity during responding (using fMRI). Higher fit participants were found to have a significant reduction in interference (as indicated by a reduced reaction time ratio between congruent and incongruent presentations), as well as increased activity in brain regions associated with attention (e.g., middle frontal gyrus, superior frontal gyrus, and superior parietal gyrus), and decreased activity in the anterior cingulate cortex, a region associated with response conflict (Colcombe et al., 2004). These results are consistent with much of the extant literature on the effects of fitness on cognitive function, particularly in older populations.

In studies utilizing an exercise intervention, similar results are observed. Colcombe et al. (2006) studied 60-79 year olds who were sedentary prior to the start of a 6-month aerobic training program or stretching and toning (non-aerobic) program. Following the respective interventions, MRI imaging showed an increase in grey and white matter volume, especially in prefrontal areas, in the aerobic exercise group, but not in the non-aerobic control participants. Acute exercise has also been shown to improve performance of a prefrontal-mediated task in both young and ageing participants. Kamijo et al. (2009) assigned both younger (19-25 years old) and older (60-74 years old) males to cycle on a stationary bicycle at either light or moderate intensity. Immediately after exercising, participants in both age groups showed shorter reaction times in a Flanker task after light or moderate intensity exercise. P3 latencies and amplitudes from event-related potential (ERP) data collected during the task also indicated changes from acute exercise. In the younger group, increased P3 amplitude was observed following moderate exercise only, while the older participants had shorter P3 latencies following either light or moderate exercise (Kamijo et al., 2009). The ERP data suggest changes specifically in processing associated with decision-making (which is linked to P3 waves).

Thinking Outside of the Hippocampus: Exercise Effects on the Prefrontal Cortex and Striatum

In an attempt to begin to bridge the gap between hippocampus-focused animal work and the human literature, our lab has focused on how exercise may benefit brain systems outside the hippocampus to change behavior in tasks that are believed to be hippocampus-independent. Set-shifting is a measure of cognitive flexibility used in both

humans and rodents. Since cognitive flexibility is one domain frequently associated with improvements following exercise in humans (Hawkins et al., 1992, Colcombe et al., 2004, Hillman et al., 2004), examining how exercise may affect performance of this task is of particular interest.

Tests of set-shifting ability can be administered to both humans and rodents, serving as a good bridge between these two research literatures. One test commonly used to assess the ability of humans to set-shift is the Wisconsin Card Sort Task. In this task, participants must sort cards based on certain stimulus rules such as color, shape, or number. Periodically throughout the test the rules are changed and the participant must inhibit responding to the previous rules and begin responding to the new rules. Individuals with disorders affecting the PFC such as schizophrenia and attention-deficit/hyperactivity disorder (ADHD) are impaired at this task in that they have a difficult time suppressing responding to the previously learned rules and continue to make perseverative responses (Green et al., 1992). One way of testing this type of executive cognitive ability in rats involves training the animal to discriminate between rewarded and unrewarded arms in a plus-shaped (four arm) maze, and then switching the discrimination rule (Stefani et al., 2003). On each trial, one of the arms of the plus maze is blocked off forming a "T", and the maze is rotated between trials. Thus, the reinforced arm is not always in a particular location in the room, nor is a particular response (e.g., right or left turn) always reinforced. This feature of the task helps rule out performance that relies upon place or response learning. The initial discrimination (set 1) is based on one of two stimulus dimensions: texture (rough/smooth) or brightness (light/dark), with

each arm consisting of a combination of the two dimensions (e.g., light and rough). In the following session (set 2), the rat is trained that the opposite stimulus dimension is relevant. For example, if the relevant stimulus dimension were brightness in the initial discrimination, with light arms being rewarded and dark arms being unrewarded, in the set-shift the relevant stimulus dimension would be texture (either rough or smooth). This requires the rat to inhibit the previously learned rule and learn a new discrimination.

Our lab has demonstrated that in adult rats, performance of the initial discrimination phase of a set-shift task is improved following two weeks of voluntary wheel running (Eddy et al., 2013). Similarly, Brockett et al. (2015) recently demonstrated that adult male rats given 12 days of running wheel access had improved object in place memory, simple discrimination, and reversal and extra-dimensional shifting performance, all tasks reliant on the mPFC and/or mPFC-hippocampus interactions. Novel object recognition, which involves the perirhinal cortex, was not affected by exercise in this study. However, Hopkins and colleagues found that adult rats that exercised for four weeks showed improved novel object recognition, along with increased BDNF in both the perirhinal cortex and hippocampus (Hopkins et al., 2011). In our experiments, rats were given ad libitum access to a locked (no exercise control) or unlocked running wheel in the home cage for two weeks total, throughout behavioral training and testing. They were given 7 total sessions of maze adaptation before set 1. In set 1, where rats must effectively discriminate between rewarded and unrewarded arms, rats that had exercised performed better (i.e., required fewer trials to reach learning criterion) than non-

exercising rats. Surprisingly, no difference between groups was observed on set 2, which tests cognitive flexibility.

These results partially fit with those from Brockett et al. (2015), in that we also saw an exercise-related improvement in a simple discrimination task (set 1). However, they also observed improvements in an extra-dimensional shift (the type of discrimination tested in our set 2) following exercise, while we did not. This discrepancy may be due to differences in experimental design (e.g., sedentary controls used by Brockett et al. had no running wheel, ours had a locked wheel to control for environmental enrichment effects; their set-shifting task utilized a foraging paradigm), rat strain used (Sprague Dawley versus Wistar), or even possibly duration of exercise (12 days versus 14). Additionally, while Brockett et al. report using adult male rats, their exact age is not indicated.

In human studies, there is evidence of cognitive processes improving following exercise interventions. Interestingly, in children and adolescents/young adults these improvements are seen in tasks relying upon prefrontal cortex function. In recent studies, both short-term and long-term exercise interventions have been demonstrated to improve executive function. Davis et al. (2011) showed that 7-11 year olds who engaged in an after school exercise program for approximately 13 weeks had improved math achievement, performance of executive function tasks, and increased PFC activity during executive function tasks. In boys (average age ~11 years old) with ADHD, daily minutes of moderate to vigorous exercise, as measured by an accelerometer, significantly

predicted performance on a planning task and increased physical activity was associated with higher scores on several measures of executive function (Gapin and Etnier, 2010).

Studies examining the behavioral changes following exercise as well as genetic differences that may mediate exercise-induced benefits have added to the understanding of potential mechanisms. Berse et al. (2015) compared performance of a switching task in adolescents (average age was approximately 15 years old) who had engaged in short bouts of cycling to those who watched a short film. Genetic polymorphisms for dopamine transporter (DAT) and dopamine receptor (D1 and D2) genes were also examined. Exercising participants were found to have a significantly lower switch cost (calculated as the differences in speed and accuracy between switch and no-switch trials) than controls (Berse et al., 2015). This difference in performance was associated with specific polymorphisms in the DAT (DAT1) and D2 receptor genes (Berse et al., 2015).

Stroth et al. (2010) also found evidence that a genetic polymorphism affecting dopamine availability is associated with exercise-related improvement in cognitive control. In participants with a mean age of approximately 23 who engaged in a running program for 4 months, increases in positive affect and cognitive control (as measured by Stroop task performance) were seen compared to controls (Stroth et al., 2010). Moreover, exercising participants who had a particular polymorphism (Val/Val genotype) in catechol-*O*-methyltransferase gene showed improvement in the Stroop task above that seen in exercisers without the same genotype (Val/Met or Met/Met), and only the Val/Val exercisers showed improvement in working memory (Stroth et al., 2010). These studies

implicate dopamine as a moderating factor in the cognitive benefits observed following physical exercise in young adults.

Our previously described set-shift data suggested that in adult rats, exercise improved performance of set 1, but not set 2. Subsequent experiments indicated that this enhancement of discrimination ability is mediated by changes to the striatal dopamine system (Eddy et al., 2014). In those experiments, rats received an infusion of a D1 or D2 receptor antagonist into the dorsolateral striatum (DLS) just before a test of discrimination ability in the same maze used for our set-shift experiments. Rats that had exercised showed improved discrimination ability. This improvement was lost when D2 receptors were antagonized, resulting in similar performance to rats that had not exercised. All non-exercising rats, regardless of infusion type (saline or D2 antagonist) performed the same. Interestingly, the performance deficit seen in non-exercise rats was rescued by an infusion of a D1 receptor antagonist, resulting in identical performance to exercising rats (while having no effect on performance of exercisers). Together, these experiments indicate that exercise modulated function and/or expression of D1 and D2 receptors in the DLS to improve performance of a discrimination task.

To investigate the somewhat surprising lack of effect of exercise on prefrontal-dependent set 2, we repeated our initial exercise and set-shifting experiments with rats of different ages. Set 2, the extra-dimensional shift portion of the task, models a behavior that is often impaired in children and adolescents with ADHD—ignoring previously learned information that has now become irrelevant and updating behavior based on new

rules. This type of perseverative, inflexible responding is associated with dysfunction of the PFC (Green et al., 1992).

Data from our lab suggest that exercise may modulate these prefrontal systems in ways similar to catecholamine re-uptake inhibitors to produce comparable behavioral changes. In juvenile (PD 30-44) male rats two weeks of voluntary wheel running or daily injections of methylphenidate (MPH) (2mg/kg, i.p.) were given leading up to set 1 and set 2 of an extra-dimensional set-shift task. MPH is a dopamine/norepinephrine (NE) re-uptake inhibitor commonly used to treat ADHD. In this experiment, rats given daily MPH or given access to a running wheel reached a learning criterion in fewer trials than rats given saline injections and locked running wheels on set 2 of the task. Subsequent experiments looked at the longevity of these effects by following the 2-week period of exercise or MPH with a break, during which running wheels were locked and no MPH was given. In this case, the data suggested that chronic MPH during development might have longer lasting effects than exercise, as only the MPH group continued to show improved performance on set 2.

We have also shown that exercise reduces expression of the norepinephrine transporter (NET), a target of MPH, in PFC. Robinson et al. (2015) demonstrated that changes in habituation of an orienting response following exercise are mediated by alterations of NE function. In those experiments spontaneously hypertensive rats (SHRs), a common rodent model of ADHD, exercised on running wheels for 2, 5, 10, or 21 days. Orienting behavior to a non-rewarded stimulus (a light) was examined. SHRs do not typically show normal habituation of the orienting response. However, following exercise

for 5, 10, or 21 days, SHRs showed identical levels of habitation to control rats. NET protein levels in the PFC were reduced after 5, 10, or 21 days of exercise, suggesting that exercise is changing noradrenergic function and that these changes might underlie normalization of the orienting response. Indeed, Robinson and colleagues showed that when propranolol, a β-adrenergic receptor antagonist, was continuously delivered systemically via an implanted pellet during the exercise period, the exercise-related change in habituation behavior was no longer present. Additionally, Dishman et al. (1997) found that chronic wheel running increased NE in the locus coeruleus (LC), the principal site of NE synthesis, which projects strongly to and receives input from the mPFC. Exercise was also associated with shortened escape latency in an uncontrollable, inescapable foot shock paradigm while in non-exercising rats, there was an escape deficit and decreased NE in the LC (Dishman et al., 1997). These data add to the growing literature suggesting that exercise causes changes outside the hippocampus, and in particular in the noradrenergic system.

NE has been shown to play an important role in learning and memory. In the PFC, phosphorylation of GluR1, which is necessary for synaptic plasticity, depends heavily upon NE (Lee et al., 2003, Pascoli et al., 2005). Patterns of activity in LC neurons have been shown to correlate with responses to target stimuli in a visual discrimination task, and patterns of firing change as performance is improved (Usher et al., 1999). LC cells that project to the mPFC, compared to other cortical LC projection sites, are more excitable and fire more spontaneously (Chandler et al., 2013), demonstrating the major influence NE has on mPFC function.

Noradrenergic projections from the LC have also been suggested to play a key role in the basic mechanisms of learning and memory by permitting burst firing in response to novel stimuli, while habituating this response quickly to familiar stimuli (Sara et al., 1994). This pattern of excitation and inhibition allows efficient and flexible use of cognitive resources by directing attention to relevant stimuli and reducing attention to background or newly irrelevant stimuli. Indeed, increased NE release in the PFC has been demonstrated to improve extra-dimensional set-shifting performance (Devauges and Sara, 1990, Lapiz and Morilak, 2006, Snyder et al., 2012). Data from set-shift and protein expression experiments from our lab suggest that exercise has behavior-modifying effects on mPFC, possibly mediated by changes in catecholamine function. However, it remains unclear if these effects are the same at different points in an animal's development. Given that the PFC is not fully developed until relatively late in life, developmental stage may govern exercise-induced plasticity in this region.

Prefrontal Cortex Anatomy and Function

The rodent PFC can be divided into three broad regions: medial, lateral, and orbital, each part with its own complex of cortical efferents and afferents (Berendse and Groenewegen, 1991, Ongur and Price, 2000). The lateral and ventral regions of the PFC include the agranular insular cortex (AI) and lateral orbital cortex (LO). These areas are primarily associated with the gustatory and olfactory systems, and are involved in forming associations between tastes and odors (Berendse and Groenewegen, 1991, Ongur and Price, 2000, Dalley et al., 2004, Kesner and Churchwell, 2011). The anterior cingulate (AC) and precentral cortex (PrCm) comprise the dorsal medial region of the

PFC and primarily have connections with sensory-motor cortical regions and project to the striatum, including the nucleus accumbens core (NAcc) (Ongur and Price, 2000, Kesner and Churchwell, 2011). Within the central and ventral medial region of the PFC are the prelimbic (PL), infralimbic (IL) and medial orbital (MO) cortices. Collectively, these sub-regions receive input from limbic and limbic association areas, including the striatum, NAc, perirhinal cortex, entorhinal cortex, hippocampus, and basal lateral amygdala while projecting to regions of the ventral medial striatum and NAcc (PL) and NAc shell (IL/MO) (Ongur and Price, 2000, Heidbreder and Groenewegen, 2003, Hoover and Vertes, 2007).

With projections centrally received at the PFC from various substrates, including regions of both cortex and sub-cortical areas including the basal ganglia, amygdala, hippocampus, and cerebellum, the PFC is ideally situated to synthesize information from various modalities and exert "top-down" control. That is, the PFC monitors sensory and motor input and influences many decision-making and attentional processes, goal-directed behavior, and working memory (Hoover and Vertes, 2007). PFC function has been demonstrated to be critical for working memory during delay tasks (Goldman-Rakic, 1990, Fuster, 1991, Li and Mei, 1994) where stimuli that are no longer physically present must be held in a mental representation in order to plan and organize behavior.

Lesions of mPFC are associated with increased perseverative responding (i.e., inability to inhibit inappropriate responses and update rule learning) and deficits in reversal tasks (Kolb, 1984, Miller, 2000). In particular, the PL and IL have been suggested to play different roles, or mediate different aspects of the same role, in domains

such as working memory for places and objects, decision making in ambiguous contexts, and cross-modal reversal learning (Heidbreder and Groenewegen, 2003, Kesner and Churchwell, 2011). Lesions of the PL result in impaired working memory, deficits in delayed response tasks, poor reversal learning, and increased perseverative responding (Floresco et al., 1997, Ragozzino and Kesner, 1998, Dias and Aggleton, 2000). Additionally, there is evidence that mPFC sub-regions play distinguishable roles in inhibition of behavior. Performance of conditioned inhibitory behavior appears to be supported by the IL, while the PL is necessary for the acquisition of the response (Meyer and Bucci, 2014).

Other sub-categories of executive function also appear to rely upon discrete regions of the PFC. Moreover, even within certain types of executive function there is evidence that PFC sub-regional involvement depends upon the specific type of task or stimulus. For example, tasks that require working memory—that is, tasks that involve holding information in short-term memory and integrating, maintaining, and retrieving such information—can rely upon different regions of PFC if they require working memory of responses versus memory of spatial locations or objects (Dias and Aggleton, 2000). For motor responses requiring working memory, lesion studies have implicated the AC and PrCm as key substrates, but they do not appear to be crucial for working memory of visual objects or spatial locations (Dias and Aggleton, 2000).

Aside from working memory tasks, there is also evidence for region-specific involvement of PFC substrates in executive function tasks such as attentional processes (including reversal learning and flexibility), decision-making, and temporal ordering of

behavior. For shifting between previously learned rules, extra-dimensional shifts and intra-dimensional shifts require separate structures, with extra-dimensional tasks relying upon PL while intra-dimensional tasks instead require functioning AI and LO cortex (Devauges and Sara, 1990, Birrell and Brown, 2000, Bissonette et al., 2013).

Development and the Prefrontal Cortex

Human PFC dysfunction is implicated in many psychopathologies, including schizophrenia, attention deficit/hyperactivity disorder (ADHD), post-traumatic stress disorder, and addiction (Gamo and Arnsten, 2011). Many of these conditions, including ADHD, schizophrenia, and drug abuse typically emerge during adolescence and early adulthood, a period marked by increased impulsivity and risk-taking behavior in both humans and rodents (Gardner and Steinberg, 2005, Kessler et al., 2005, Brenhouse and Andersen, 2011).

Maturation of the PFC is relatively late compared to other cortical regions, with full maturity not achieved until late in adolescence/early adulthood, which in humans occurs at about 20-25 years of age and at approximately postnatal day (PD) 42-50 in rodents (Spear, 2000). At this developmental time point, cortical volume and dendritic spine density become stabilized (van Eden et al., 1990, Marmolejo et al., 2012). Considerable over-production of synapses in the PFC occurs early in development (as well as a corresponding increase in PFC volume), which peaks around PD24-30 (van Eden et al., 1990). The peak in PFC volume and dendritic spines/complexity is then followed by a relatively drawn out period of pruning until approximately PD60 (Van Eden and Uylings, 1985, van Eden et al., 1990, Marmolejo et al., 2012). This pruning and

accompanying decrease in PFC volume presumably serve to refine desirable synapses and support beneficial connections, while removing unnecessary ones, increasing efficiency as the PFC matures. Figure 1 summarizes stages of brain development in rats and humans.

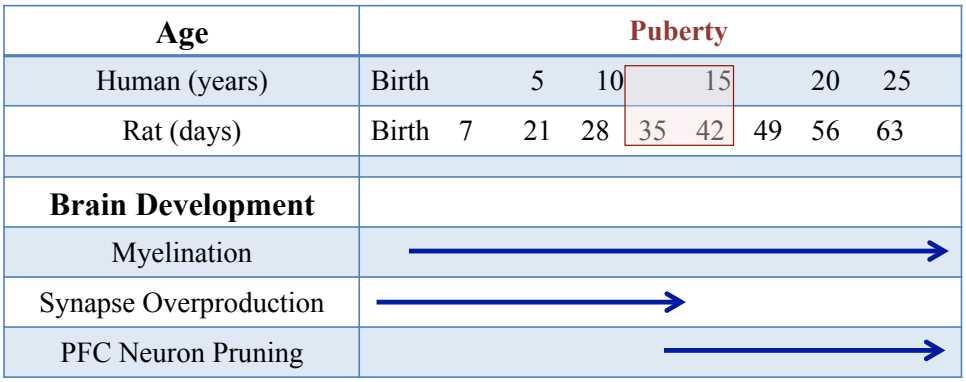

Figure 1. Timeline of brain development in rats and humans (modified from Kolb et al., 2012).

Because the PFC reaches final maturity so late in development, this permits other brain regions, which have already fully matured, to modulate its development. Early sensory and motor experience (impoverished or enriched) has been shown to have profound effects on developmental outcomes. For example, rat pups given tactile stimulation for 18 days (PD3-21) show improved novel object recognition and motor skills (measured by Wishaw tray reaching task), coupled with increased dendritic length, branching, and spine density in PFC compared to non-stimulated litter mates in adulthood (approximately 100 days old) (Richards et al., 2012). Together with a substantial amount of data showing that early life exposure (ranging from prenatal to PD14-40) to drugs, stress, or other negative environmental factors have long lasting consequences on brain

structure and function (Kjaerby et al., 2014, Proulx et al., 2014, Liu and Crews, 2015, Skorput et al., 2015), early exposure to environmental enrichment is clearly important.

Indeed, there is evidence that as rats age the mPFC becomes less receptive to experience-related plasticity. Kolb et al. (2003) examined pyramidal cells of the nucleus accumbens and PFC (specifically, Cg3 region) in adult (90 day old) female rats that lived in either standard housing or in an enriched environment (which included access to toys, objects to climb on, etc.) for their entire lives. Rats housed in the enriched environment had increased dendritic length, branching, and spine density in the nucleus accumbens, but only increased spine density (i.e., no increased dendritic length or branching) in the PFC (Kolb et al., 2003). While this result does suggest that experience with a complex environment can induce prefrontal plasticity, the fact that there was relatively less change in the PFC compared to nucleus accumbens may highlight the importance of early-life exposure to positive experiences and environmental stimuli.

Behaviorally, improvements are seen in tasks mediated by the PFC from adolescence to adulthood. In humans, executive function (including working memory, planning, cognitive flexibility and shifts in attention) normally improves as adolescences advances into adulthood (Anderson et al., 2001, De Luca et al., 2003, Gardner and Steinberg, 2005). Likewise, rodent studies have demonstrated that age-related changes in the PFC underlie alterations in risk-taking behaviors (Brenhouse et al., 2008). Around adolescence, D1 receptor expression in PFC is increased significantly, and has been suggested to play a role in the enhanced proclivity toward addictive behaviors by enhancing prefrontal D1 receptors' signaling in the PFC-nucleus accumbens pathways

(Brenhouse et al., 2008). By infusing SCH23390, a D1 receptor antagonist, into the PFC of adolescent rats, Brenhouse et al. (2008) showed that when this receptor subtype is effectively taken "off-line" the enhancement in cocaine-induced conditioned place preference normally observed in adolescent rats is blocked.

NE transmission in PFC-limbic circuitry has also been shown to be a critical component of cognitive control during development. In adolescent rats (PD35) infusion of MPH into AC, IL, basolateral amygdala or habenula inhibited social play, a behavior that is important for social and cognitive development (Achterberg et al., 2015). Indicating that this effect was due to the actions of MPH on NE, infusions of atomoxetine, a NE re-uptake inhibitor, in the same brain regions also produced an inhibition of social play. The authors suggest that because a decrease in social play in young rats indicates more behavioral inhibition, these drugs, by acting on the prefrontal-limbic pathway NE-neurotransmission can facilitate cognitive control (Achterberg et al., 2015).

The Developing Prefrontal Cortex and Pavlovian Conditioning

Mechanisms involved in Pavlovian conditioning paradigms also appear to change as animals develop. Fear conditioning in particular has been shown to be fundamentally different in very young rats compared to young or adult rats. In a typical fear conditioning paradigm, a neutral stimulus (e.g., a light or tone) is repeatedly paired with a negative outcome, such as a shock. This results in the formation of an association between the previously neutral stimulus (conditioned stimulus, CS) and the negative event (unconditioned stimulus, US). The learned association then elicits a behavioral

response, the conditioned response (CR), such as freezing, in response to the CS. This type of learning requires relatively few trials and can be observed across species, including humans.

This acquired behavior can be suppressed by presenting the CS repeatedly in the absence of the US in a procedure called extinction. Extinction results in a reduction of the CR, which importantly does not represent forgetting of the original leaning, but rather new learning about the previously fear-eliciting stimulus—sometimes it results in a negative outcome, while other times it does not. In essence, the CS now has two possible meanings, and its meaning depends on the context in which it is experienced. Indeed, we now appreciate that extinction is a particularly context-dependent type of learning and is not erasure of the original CS-US association (Bouton, 2004, Bouton et al., 2006). This concept is supported by several phenomena that result in the re-emergence or recovery of extinguished behavior, including reinstatement, renewal, rapid reacquisition, and spontaneous recovery.

Studies of the role of the PFC in conditioning at different developmental time points in rats have revealed differences in fear conditioning depending on the age of the animal. Adolescent rats (PD35), but not pre-adolescent (PD24) or adult (PD 70) rats show impaired retention of extinguished fear conditioning 24 hours after extinction (McCallum et al., 2010), suggesting that the normal pruning of PFC neurons that takes place during adolescence may impair recall of extinction. Similar results add evidence to the idea that the role of the PFC may change across development. In very young, pre-weaning rats the PFC is not necessary for extinction, while in post-weaning (PD24) and

older rats the PFC is necessary for extinction, as well as recovery effects such as reinstatement, renewal, and spontaneous recovery (Kim and Richardson, 2010). Kim et al. (2009) found that temporary inactivation of the vmPFC using muscimol (GABA agonist) prior to extinction training (that is, the mPFC was inactivated during all extinction training) resulted in impaired expression of extinction in PD24 rats, but not PD17 rats. Additionally, cells expressing phosphorylated mitogen-activated protein kinase (pMAPK), a signaling pathway involved in many regulatory processes including proliferation, apoptosis, and gene expression, were increased in the IL and BLA of PD24 rats, but only increased in the BLA of PD17 rats, suggesting that while the mPFC (in particular, IL) and amygdala are necessary for extinction retention/recall in PD24 rats, only the amygdala is involved in extinction of fear conditioning in PD17 rats (Kim et al., 2009). These studies suggest that, at least in Pavlovian fear conditioning, the underlying neural mechanisms involved in extinction may change as an animal develops. Indeed, there is evidence to support the idea that early exposure to stress can modify the development of fear extinction. Maternal separation early in life (during the first two weeks of life) results in adult-like fear extinction, with improved extinction and increased renewal and reinstatement compared to non-stressed animals (Callaghan and Richardson, 2011, Callaghan and Richardson, 2012).

Renewal of Extinguished Instrumental Responding

Conditioned instrumental (operant) behaviors are voluntary actions that are controlled by their consequences. Animals readily acquire behaviors (e.g., lever pressing) to obtain a desirable outcome (e.g., food pellet or drug delivery) and likewise learn to

suppress behavior when the reinforcer is withheld; this is referred to as extinction. Extinction of instrumental responding is an important and fundamental component of behavioral change (Bouton, 2014; Bouton & Todd, 2014). Extinguished behaviors can re-emerge through several associative mechanisms including reinstatement, resurgence, renewal. Renewal of an extinguished instrumental behavior occurs when an animal is exposed to a context that is different from the extinction context (e.g., ABA, where A and B refer to different contexts) (Bouton et al., 2011). This re-emergence of the extinguished behavior upon removal from the extinction context demonstrates that extinction is not erasure of the original learning, but rather new learning that inhibits the originally acquired association—and that this new learning is particularly context dependent. This new learning includes two available meanings about the acquired behavior: that sometimes it predicts a reward, while other times it does not. The "meaning" is determined by the context, which can be excitatory, as it was in acquisition, or inhibitory, as in extinction. Indeed, it appears that instrumental extinction is a context-specific type of inhibitory learning in that the extinction context comes to signal that no reward is available and suppresses responding *in that particular context* (Bouton, 2004, Bouton et al., 2011). These two memories of response-outcome associations are maintained concurrently by the animal and selected based on the circumstances or context in order to maximize achievement of some goal (e.g., obtain food reward). Extinction is more sensitive to context than acquisition—that is, associations formed during acquisition generalize between contexts more readily than the inhibitory associations formed in extinction, which is demonstrated in preparations that demonstrate renewal without return

to the acquisition context. In situations where acquisition and extinction occur in context A, renewal is still evident when tested in context B (AAB) or when acquisition, extinction and test all occur in novel contexts (ABC) (Bouton, 2004, Bouton et al., 2011). Thus, simply removing the inhibition from the extinction context is sufficient to elicit a return of responding.

Instrumental renewal has been reliably demonstrated with several different reinforcers and across different paradigms, though it has been studied considerably less thoroughly than renewal of Pavlovian responses. ABA renewal (conditioning in context A, extinction in context B, testing in context A) has been shown with food reinforcers (e.g., Nakajima, Tanaka, Urushihara, & Imada, 2000) and different drug reinforcers (e.g., alcohol, cocaine, heroin) (Bossert et al., 2004, Fuchs et al., 2007, Hamlin et al., 2008, Chaudhri et al., 2009). Additionally, AAB and ABC renewal have also been observed in instrumental conditioning (Bouton et al., 2011) and show that removal from the extinction context is sufficient to elicit renewal of responding; return to the acquisition context is not necessary. Such results suggest that extinction at least partly involves learning to inhibit the response in the extinction context.

Behavioral and Neurobiological Mechanisms of Instrumental Renewal

Previous work has provided us with substantial information about the learning mechanisms underlying instrumental extinction and renewal. Bouton et al. (2011) showed that instrumental renewal can be observed in ABA, AAB, and ABC paradigms. There are several possible behavioral mechanisms that may underlie renewal in these different forms (e.g., Bouton, 1993). Experiments by Bouton et al. have demonstrated that removal

from the extinction context is sufficient for renewal to occur. This has been suggested to at least in part be due to a removal of inhibition. During acquisition, an excitatory association can be formed between the action (e.g., lever press) and reward (Todd et al., 2012a, Todd, 2013, Todd et al., 2014). When extinction then takes place in a separate context, a new association is presumably learned: that in this particular context, the same action no longer results in reward.

Additionally, renewal also occurs when the associative histories of the extinction and renewal contexts are equated (see Todd, 2013; Todd et al., 2014b). Instead of being dependent upon context-reinforcer associations, Todd (2013; see also Todd et al., 2014b) suggested that renewal is at least partly due to the removal of context-specific response inhibition when the animal is tested outside the extinction context (cf. Rescorla, 1993, 1997). Previous extinction of the same response in the renewal test context reduces renewal (Todd et al., 2014b), whereas previous extinction of a different response does not (Todd, 2013; Todd et al., 2014b). The latter result appears inconsistent with an occasion-setting mechanism, in which the context might signal or set the occasion for a response-no-outcome relationship, because the power of occasion setters is expected to transfer between similarly trained targets (e.g., Holland, 1992). Extinction may thus instead result from direct inhibition of the specific response.

The PFC, given its role in monitoring and selecting appropriate behaviors, while inhibiting inappropriate behaviors, is a likely candidate to play a role in response selection in extinction and renewal. There is a relatively small literature looking at the role of the mPFC in operant renewal, though it appears probable that sub-regions of the

mPFC play opposing roles in promoting and suppressing behavior. It is often assumed that the PL and IL regions of the mPFC exert control over behavior in separate ways, with the PL driving expression of behavior, and the IL suppressing behavior. This type of functional separation has been suggested in studies using various reward seeking paradigms. Fuchs et al. (2005) found that renewal ("context-induced reinstatement") of lever pressing for cocaine does not necessarily rely upon the same substrates as reinstatement ("primed reinstatement", using a priming dose of cocaine). Using tetrodotoxin to inactivate the dorsal mPFC, dorsal hippocampus, or basal lateral amygdala (BLA), they demonstrated that the dorsal mPFC is necessary for both ABA renewal and reinstatement, while inactivation of the dorsal hippocampus or BLA eliminated ABA renewal only (Fuchs et al., 2005). Conversely, inactivation of ventral (but not dorsal) mPFC decreased expression of renewal while activation (indicated by cFos expression) of both the ventral and dorsal mPFC is associated with a return to context A in an ABA design in which rats lever-pressed for heroin infusions (Bossert et al., 2011). Bossert et al. (2012) also demonstrated that cFos activation in the ventral mPFC and its projections to the nucleus accumbens shell promote renewal of heroin seeking.

The disparity in these findings suggests that the reinforcer (e.g., cocaine versus heroin) and possibly the response (lever-pressing versus nose-poking) may affect the specific roles ventral and dorsal mPFC structures play in renewal. Willcocks and McNally (2013) found that inactivation of the PL reduced expression of renewal of extinguished nose-poking for alcohol. That is, when returned to the acquisition context

(A), animals with inactivated PL cortices did not show the large increase in nose-poke responding for alcoholic beer (compared to the extinction context, B) seen in control rats. Cognitive control over behaviors such as responding in extinction and renewal clearly has mPFC underpinnings, though the precise role of the mPFC sub-regions (specifically the PL and IL) in renewal of instrumental behavior is still unclear.

Context-specific inhibitory learning is an important element of extinction. As discussed above, the PL and IL regions of the mPFC have been shown to play critical, and possibly opposing roles in operant extinction and renewal in tasks using drug reinforcers (McFarland and Kalivas, 2001, Fuchs et al., 2005, Fuchs et al., 2007, Peters et al., 2008, Bossert et al., 2011, Bossert et al., 2012, Peters and De Vries, 2013, Willcocks and McNally, 2013). Yet, the precise roles of these regions in extinction and renewal of responding for a non-drug, food reward are not certain.

The experiments presented here draw upon several well-established methodologies to explore the behavioral and physiological changes following exercise in adulthood compared to adolescence, as well as the role of mPFC sub regions in instrumental extinction and renewal. To that end, experiments examining instrumental renewal of extinguished responding for a food reinforcer following exercise or pharmacological manipulations were carried out. These experiments provide evidence of the specific roles of the PL and IL in instrumental renewal when a food pellet is used as the reinforcer. Support for the hypothesis that physical exercise has effects on the mPFC of juvenile rats, but not adults, is presented. Additionally, the underlying cellular and morphological changes that coincide with exercise intervention were evaluated, providing

evidence that physical exercise does indeed result in not only modifications of behavior, but also the underlying neurobiology. Together, this research fills a gap in the body of literature on how exercise affects the neocortex, and specifically how its effects may be limited by the developmental state of the animal. Further, novel results are presented to demonstrate the individual contributions of PL and IL mPFC to instrumental renewal using a food reinforcer.

EXPERIMENT 1: EXERCISE AND ABA RENEWAL (JUVENILE RATS)

Experiment 1 was designed to examine how exercise during development affects expression of extinction and ABA renewal in an appetitive lever-pressing paradigm. Because our data from set-shift experiments using juvenile rats suggest changes specifically in the mPFC, I predicted that exercise would have an effect on instrumental renewal, as it also has mPFC underpinnings (McFarland and Kalivas, 2001, Fuchs et al., 2007, Bossert et al., 2011, Bossert et al., 2012, Willcocks and McNally, 2013). Reducing renewal is a topic of particular interest in the context of maintaining behavior change. One type of behavior change that is especially challenging to treat addiction. Lapse and relapse, which involve a return of the unwanted behavior upon context change, are major barriers in treating pathological drug taking, eating, gambling and other habitual behaviors.

How the rodent mPFC as a whole and its sub-regions are potentially affected by exercise, particularly during development, has until this point largely gone unstudied. Moreover, how exercise-induced plasticity may influence extinction and renewal remains unknown. The improvement in set-shifting performance that we observed in exercising juvenile, but not adult, rats suggests that the developing mPFC may be especially primed to benefit from exercise. Therefore, I predicted that juvenile rats that exercised for two weeks would differ in level of extinction and/or renewal exhibited compared to rats of the same age that had not exercised.

Methods

Subjects. Male Wistar rats obtained from Charles River Canada were used. Juvenile rats were 21 days old upon delivery, and were initially housed in groups of 4. Animals were housed in a colony room that was temperature and humidity controlled, and kept on a 12/12 hr light/dark schedule. At the start of the experiment (PD30) rats were individually housed in cages equipped with a locked or unlocked running wheel (described below). All rats were given at least 5 days of acclimation in the colony following their arrival, during which time they had ad libitum access to food and water. Prior to magazine training, rats were food restricted to approximately 85-90% of their free feeding weight. Target weights were determined by taking 85% of the projected normal weight at the appropriate point in the growth curve for non-restricted male Wistar rats.

Voluntary Exercise. Rats assigned to the exercise group were given unlocked running wheels following colony acclimation. Non-exercise animals were given identical wheels that were locked in place to control for environmental enrichment effects. Rats had 24-hour ad libitum access to running wheels for approximately 14 days total. Rats had wheel access throughout the experiment, including testing. The running wheels (Med Associates Inc., St Albans, VT) were 36 cm in diameter and had an automatic counter attached that recorded every quarter revolution. Wheel counts were taken at the same time each day.

Apparatus. Two sets of four operant chambers were used in these experiments, which served as context A and context B (counterbalanced across groups). The chambers

were slightly modified versions of Med Associates (St. Albans, VT) model ENV-008-VP chambers. They measured 30.5 x 24.1 x 21.0 cm (l x w x h) and were individually housed in sound attenuation chambers.

In one set of boxes the sidewalls and ceiling were made of clear acrylic plastic, while the front and rear walls were made of brushed aluminum. The floor was made of stainless steel grids (0.48 cm diameter) staggered such that odd- and even-numbered grids were mounted in two separate planes, one 0.5 cm above the other. A recessed 5.1 x 5.1 cm food cup was centered in the front wall approximately 2.5 cm above the level of the floor. Retractable levers were positioned to the left and right of the food cup. These levers were 4.8 cm long and were positioned 6.2 cm above the grid floor. The left lever protruded 1.9 cm when extended (the right lever remained retracted over the course of the experiment). A pair of 28-V panel lights (2.5 cm in diameter) was attached to the wall 10.8 cm above the floor and 6.4 cm to both the left and right of the food cup. These chambers were illuminated by two 7.5-W incandescent bulbs mounted to the ceiling of the sound attenuation chamber, approximately 34.9 cm from the grid floor at the front wall of the chamber. Ventilation fans provided background noise of 65 dB. This set of boxes had no distinctive visual cues. A glass dish containing lemon scented cleaner was placed outside of each chamber near the front wall.

The second set of boxes was similar to the lemon-scented boxes (also model ENV-008-VP, with similar placements of levers and panel lights), except for the following features. In the second set of boxes, one sidewall had black diagonal stripes, 3.8 cm wide and 3.8 cm apart. The ceiling had similarly spaced stripes oriented in the

same direction. The grids of the floor were mounted on the same plane and were spaced 1.6 cm apart (center to center). These chambers were illuminated by one 7.5-W incandescent bulb mounted to the ceiling of the sound attenuation chamber, near the back wall of the chamber. A distinct odor was continuously presented by placing pine scented cleaner in a glass dish outside the chamber. Both sets of boxes delivered a 45-mg sucrose pellet (TestDiet, Richmond, IN, USA).

Procedure. On day 1, magazine training, rats learned to retrieve sucrose pellets from the food cup. Pellets were delivered on average every 30 seconds. Rats underwent two separate 30-minute sessions in both contexts A and B. During this session there was no lever present. Following magazine training, rats underwent 6 daily sessions of lever training (acquisition) in context A. During these sessions, following a two-minute interval the left lever was inserted and a VI-30 reinforcement schedule was in effect. Each session lasted 30 minutes.

Following acquisition, extinction occurred in the opposite context (context B). All parameters were identical except that a lever press no longer resulted in delivery of a sucrose pellet. Extinction took place over 4 daily sessions. On the day after the final extinction session, all animals were tested in a within-subject manner. Each rat was tested in two separate 10-minute sessions, one in context A and one in context B, in a counterbalanced order. As in extinction sessions, after 2 minutes the left lever was extended but lever presses did not result in a sucrose pellet. The two test sessions were separated by approximately 30 minutes. In all sessions, lever presses, magazine entries,

and pellets obtained were counted. Following each daily session rats were returned to the home cage and fed approximately 10-20g of food.

Results

Two weeks of exercise in adolescent rats (i.e., 30-45 days old) reduced renewal of responding. In acquisition and extinction of lever-pressing behavior, no differences were seen between exercising and non-exercising rats. When tested for renewal, both groups showed significant renewal of responding (i.e., greater responding in the non-extinction context A compared to the extinction context B), $F(1,28) = 85.26$, $p < 0.01$. However, exercising rats showed significantly less renewal than non-exercising rats, $F(1,28) = 4.87$, $p < 0.05$ (Figure 2). When test data were divided into 30 second bins, it was clear that exercising rats were responding less almost immediately during the test session (repeated measures ANOVA, $F(1,14) = 31.16$, $p < 0.01$; significant bin x group interaction $F(1,14) = 31.16$, $p < 0.05$), with significant differences between the two groups in the first 6 bins (Figure 3).

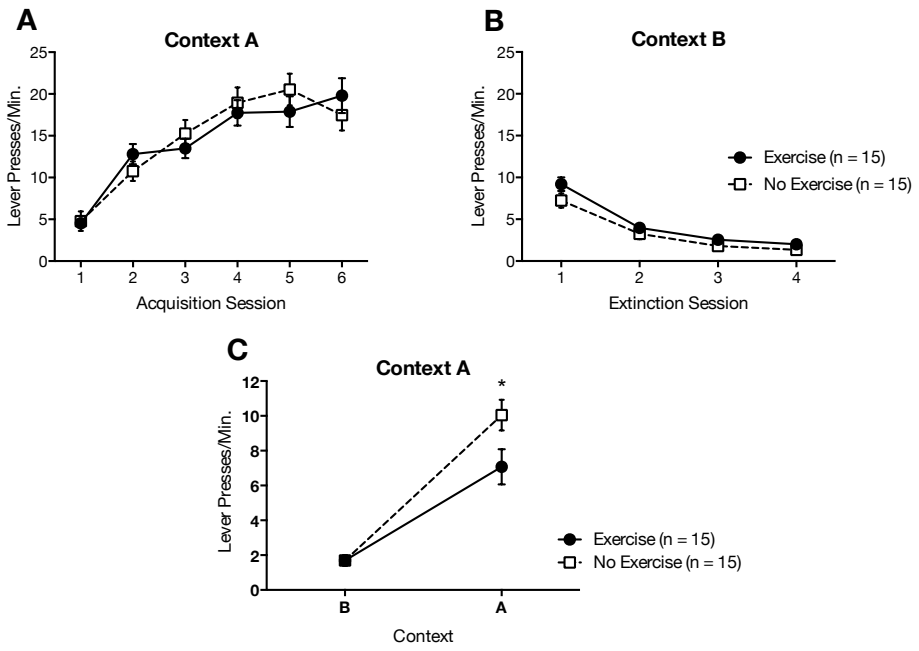

Figure 2. Lever press rates of juvenile rats that had access to a locked or unlocked running wheel for two weeks. Acquisition took place in context A and extinction in context B. There were no differences in responding during acquisition (panel A) or extinction (panel B). Both groups showed renewal of responding (increased responding in context A compared to context B) while exercising rats showed significantly less renewal than non-exercising rats (panel C).

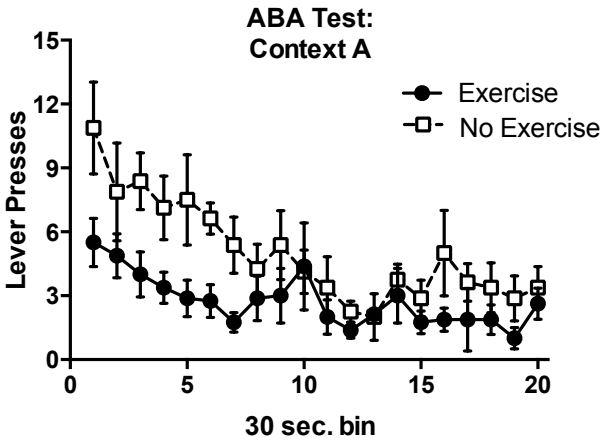

Figure 3. Lever presses made in context A during 10-minute test session in experiment 1 divided into 30-second bins. Exercise rats responded significantly less than non-exercise rats in the first 6 30-second bins.

Discussion

In this experiment, two weeks of exercise during development reduced renewal in an ABA paradigm. Importantly, renewal of responding in exercising rats was still present upon return to context A. This indicates that exercise is modulating some component of renewal and/or extinction, but is not abolishing renewal. Several possibilities may explain this reduction of renewal.

It does not seem that this difference is due to improved context discrimination by exercisers, as there were no differences observed in rate of extinction, which took place in a different context (B) from acquisition (A). While it is conceivably possible that at the start of extinction rats had not had enough exposure to exercise (around 10 days) for there to be an effect, this seems very unlikely. Another possibility is that exercising rats may have better inhibitory control of their behavior. Indeed, when test data are divided into 30-second bins there is support for this hypothesis. One would expect to see initial responding when the lever is made available in context A, since up to this point lever pressing in this context has resulted in reward. However, because of the absence of reward in this context during test, a new, inhibitory association between context A and the response will form, and lever pressing should decline (Todd, 2013). The 30-second bin data suggest that exercising rats may be forming this inhibitory association faster, as they suppress responding more quickly than non-exercising rats. This explanation is consistent with the improvement observed in set-shifting in juvenile rats after exercise.

EXPERIMENT 2: EXERCISE AND AAB RENEWAL (JUVENILE RATS)

To examine these possibilities, a follow-up experiment was run using the same methods and preparation as described above, except that acquisition and extinction occurred in the same context (A). Because extinction is slowed when it occurs in the acquisition context, animals were run for an additional 2 sessions (6 total), at which point they reached similar levels of responding to those observed in experiment 1. Rats were then tested in a new context, B. This paradigm allows dissociation between improved generalization of extinction learning and improved consolidation/retrieval of extinction by introducing a novel context at test. If exercise results in better consolidation of extinction, then no reduction of AAB renewal would be expected.

Methods

Methods for experiment 2 were identical to experiment 1 except that acquisition and extinction sessions occurred in the same context (context A), and all rats were given two additional extinction sessions (6 total).

Results

Non-exercising rats showed significant AAB renewal $F(1,11) = 9.77$, $p = 0.01$, while exercising rats did not show a significant increase in responding when exposed to context B (no renewal of responding). The difference between groups in context B responding was significant $F(1,22) = 8.07$, $p < 0.01$, as was the group by context

interaction $F(1,22) = 4.49$, $p < 0.05$ (Figure 4). The average daily running distance for rats is shown in Figure 5.

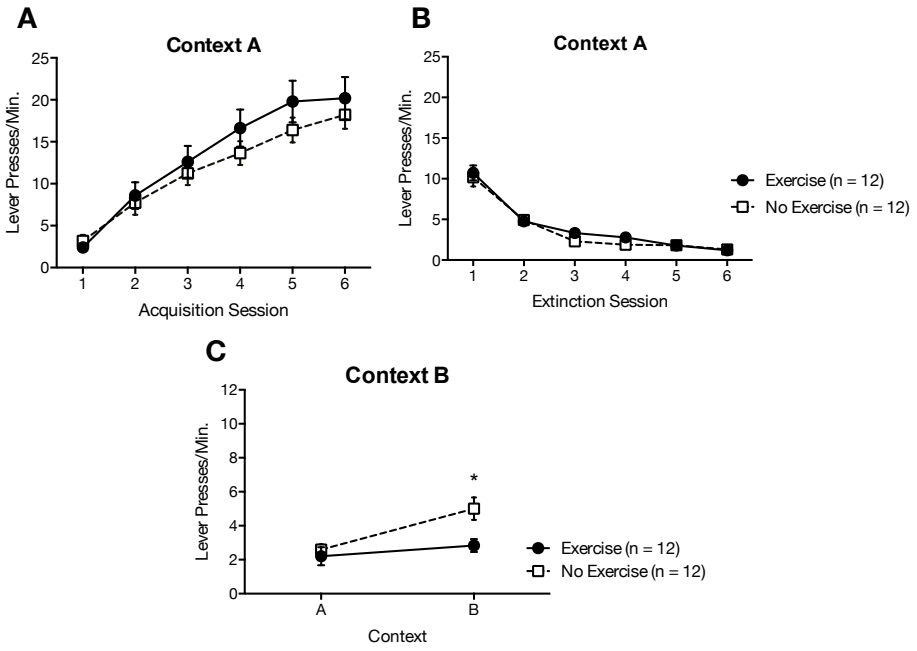

Figure 4. Lever press rates of juvenile rats that had access to a locked or unlocked running wheel for two weeks. Acquisition and extinction took place in the same context (context A). There were no differences in responding during acquisition (panel A) or extinction (panel B). Only non-exercise rats showed renewal of responding (increased responding in context B compared to context A). Rats that had exercised showed no renewal (no change in responding in context B compared to context A) (panel C).

Discussion

This experiment expands upon the first by eliminating one possible mechanism through which exercise could be reducing renewal—that exercise is improving consolidation of extinction learning. No significant increase in responding between

context A and context B was seen in exercising rats. This suggests that there was no renewal of responding. However, because the AAB renewal effect is subtler than ABA, this may be due to a floor effect. AAB and ABC renewal effects show a relatively smaller return of responding when compared to ABA (Bouton et al., 2011). This is likely because in ABA, animals generalize more between the acquisition context and the test context, since 100% of the elements comprising the acquisition context are present at test, which presumably results in maximum retrieval of acquisition learning (Todd et al., 2012b). In AAB and ABC paradigms the recovery of responding is due to a removal from the extinction context, and while the test context may share some contextual elements with the acquisition context, there will be a smaller proportion of common elements that are available to activate learning from the acquisition or extinction contexts.

Indeed, here renewal was seen in non-exercising rats when they were tested in a context different from the acquisition and extinction contexts. These data suggest that exercising rats may be better at inhibiting their behavior. At the beginning of the test, context B has no direct association with lever-pressing. We would expect to see responding, as the response (lever press) has not been extinguished in this context and because of generalization between contextual elements (e.g., presence of the lever) in this context. However, as the context enters into an inhibitory association with the response (due to the lack of reward), responding should decline as the animal stops generalizing between the stimuli and associations formed in acquisition and those that are present in extinction (Todd et al., 2012b, Todd, 2013). It appears that exercising rats may be forming this inhibitory, CS-no US association faster than non-exercising rats, resulting in

a suppression of responding more quickly than non-exercising rats. Alternatively, exercisers may generalize less between contexts (that is, they are more sensitive to changes in elements of the context). If this were the case, then context B will elicit less retrieval of the original excitatory learning than context A and result in less renewal.

EXPERIMENT 3: EXERCISE AND ABC RENEWAL (JUVENILE RATS)

To further examine the renewal effects seen in the first two experiments, a third experiment examined ABC renewal. ABC renewal differs from AAB renewal in that each phase takes place in a new context. This is a potentially important distinction because in AAB, extinction occurs in the same context as acquisition, while extinction occurs following a context switch in ABC. If exercising rats are discriminating between elements of the contexts better than non-exercisers, and thus adjusting their responding more quickly, even less renewal in ABC would be predicted. In this type of preparation, generalization between the acquisition and extinction contexts should be equal to that seen in ABA (the two contexts share the same number of shared elements). If exercisers are better able to discriminate between contexts based on distinct elements, then there will be even less excitatory influence from the learning that took place in context A (since, as mentioned above, acquisition learning generalizes more strongly between contexts than extinction learning) when they are exposed to a novel context. That is, there will be more generalization decrement in ABC than there was in ABA or AAB. The purpose of this experiment was to determine if the same reduction of renewal following exercise would be seen when acquisition, extinction and test for renewal all occur in novel contexts. In both AAB and ABC preparations, simply removing the animal from the extinction context produces renewal of extinguished responding (Todd et al., 2012a), demonstrating the dependence of extinction upon context. I expected that in this experiment there would continue to be differences between exercising and non-exercising

juvenile rats. If exercise improves the ability of the animals to discriminate between contexts based on common stimuli (e.g., the lever CS) and distinct stimuli, then the prediction would be low responding in context C compared to non-exercisers. As ABC renewal has been reliably demonstrated for extinguished instrumental responding for a food reinforcer (Bouton et al., 2011), I expected to see a recovery of responding in context C compared to context B.

Methods

Methods and materials for experiment 3 were identical to those used in experiments 1 and 2, except that acquisition, extinction, and test all occurred in different contexts (ABC). One animal from the non-exercising group was excluded because it did not reach extinction criterion. Data from a total of 23 rats (12 exercise, 11 no exercise) was included in final analyses.

Two of the contexts were the same set of boxes (pine and lemon) used in experiments 1 and 2. The third context was similar to the pine and lemon-scented boxes (also model ENV-008-VP, same placement of lever, etc.) except that the grids of the floor were mounted on the same plane and spaced 1.6 cm apart. The chambers in context C were illuminated by one 7.5-W incandescent bulb mounted to the ceiling of the sound attenuation chamber. An odor cue was presented by placing a small glass dish containing a small amount of vinegar just outside the chamber. These three sets of boxes served as contexts A, B, and C in a counterbalanced design. All animals were exposed to each of the contexts during magazine training, which took place during a 30-minute session

where pellets were delivered in a non-contingent manner (the lever was not present during this session). Context order at test was fully counterbalanced.

Results

Two weeks of exercise in adolescent rats (i.e., 30-45 days old) had no effect on renewal of responding when tested in a novel context (C). In acquisition and extinction of lever-pressing behavior, no differences were seen between exercising and non-exercising rats. When tested for renewal, both groups showed significant renewal of responding (i.e., greater responding in C compared to the extinction context B), $F(1,21) = 5.92$, $p = 0.02$. However, there was no significant difference between groups in responding in context B or context C (Figure 5). Average daily distance run is shown in Figure 6.

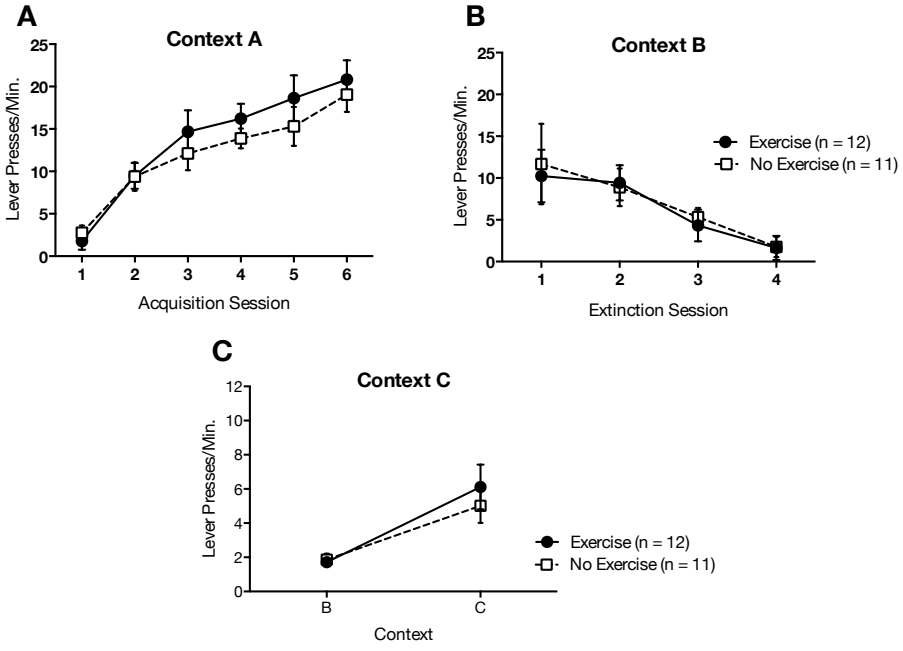

Figure 5. Lever press rates of juvenile rats that had access to a locked or unlocked running wheel for two weeks. Acquisition took place in context A, extinction in context B, and test in context C. There were no differences in responding during acquisition or extinction. During the renewal test,

both groups showed renewal of responding (increased responding in context C compared to context B) and did not differ from one another.

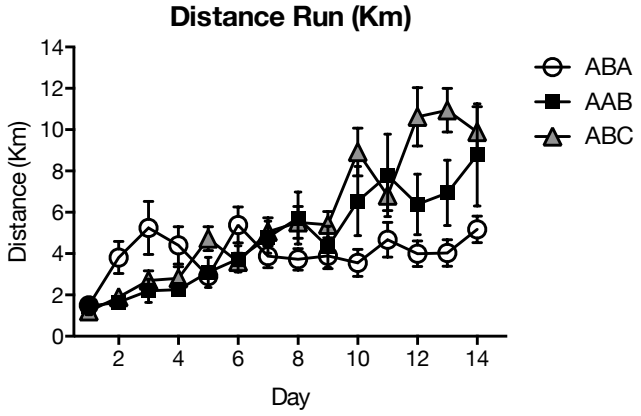

Figure 6. Average daily distance run for juvenile rats from experiments 1, 2, and 3.

Discussion

Unlike other types of renewal, exercise did not have an effect on ABC renewal in this experiment. This result was somewhat surprising, considering there was an effect on AAB renewal. It is unlikely that this lack of effect is simply due to a floor effect, as AAB and ABC renewal are of similar magnitude, and exercise did have an effect on AAB. Also unlikely is an effect of the distance run by the rats in experiments 2 and 3. While on average overall rats in experiment 3 (ABC) did run slightly more than those in experiment 2 (AAB) (average daily distance = 4.69 km and 5.72 km for experiments 2 and 3, respectively) there is no substantial evidence in the literature to suggest that the amount of running has an effect on the strength of exercise-related changes.

Theoretically, there may be a difference in the underlying mechanisms through which behavior re-emerges in AAB and ABC renewal, even if the magnitude of the

effects is similar. In ABA, renewal is numerically largest. This can be attributed to the fact that a return to the context where the original learning took place is very effective at eliciting that behavior learned in acquisition. When extinction occurs in context B, the CS (lever), which is common to both contexts A and B, now enters into a new association—that in this particular context, it no longer predicts the availability of reward. This new learning is maintained along with the original learning. When the rat is then re-exposed to context A, which shares 100% of elements of the context in which acquisition took place, it elicits a strong retrieval of the original excitatory learning, and thus a robust return of responding. If we assume the same basic mechanisms occur across all types of renewal, then in AAB the acquisition and extinction contexts share 100% of the contextual elements, except the availability of the US. The absence of the reinforcer is a change in the stimuli that were present during acquisition and extinction, and this can reduce responding. This generalization decrement reduces responding, but there is also active inhibition of the acquisition learning by the newly formed CS-no US association (Bouton, Winterbauer, & Vurbic, 2011). Because we know that extinction learning is more sensitive to changes in context than acquisition learning, then when the rat is placed in a novel context, we would expect more generalization of the learning that occurred in acquisition to this context, which shares some of the same contextual stimuli, and thus an increase in lever-pressing. In ABC, there are some common elements in the associations formed in A (acquisition) and B (extinction). Therefore, a smaller proportion of elements from context C will generalize to A or B. If there is more generalization decrement in ABC than in AAB, then it is possible that this may reduce the amount of new inhibitory

learning that occurs in extinction. If this holds true, then the lack of effect of exercise on ABC renewal could conceivably be due to the difference in inhibitory learning taking place during extinction in AAB versus ABC. New inhibitory learning may be improved by exercise, resulting in less renewal in AAB, but if generalization decrement in ABC reduces the amount of inhibitory learning taking place, exercise may not have a noticeable effect. To examine this possibility, an experiment could be run to minimize generalization decrement in ABC. One possible approach could be to present reinforcers in a non-contingent manner during extinction. This would eliminate one difference between the shared elements experienced in context A and B, and reduce the impact of generalization decrement, thus allowing for more inhibitory learning.

EXPERIMENT 4: EXERCISE AND ABA RENWAL: COMPARISON OF ADULTS AND JUVENILES

The purpose of this experiment was to replicate experiment 1, but to add a comparison with adult rats. This is an important experiment for two reasons: first, to demonstrate replicability of the exercise effect on ABA renewal in young rats; and second, to see if exercise affects ABA renewal in adults. A reduction of ABA renewal in exercising juveniles (as previously observed) but not in adults may suggest that there is a critical developmental period during which the mPFC is amenable to plasticity induced by exercise. There are some data to suggest that plasticity (indicated by alterations in dendritic spines in response to stress or normal aging) of the mPFC may vary across development (Bloss et al., 2011, Dickstein et al., 2013, Anderson et al., 2014), though whether exercise's effects may differ due to changes in plasticity remains unknown. If both exercising adults and juveniles show reduced renewal, then this may suggest that mPFC circuitry is malleable into adulthood. Based upon our previous data, I predicted that exercise would reduce ABA renewal in juvenile rats only.

Methods

Methods were identical to experiment 1 except that in addition to the two groups of juveniles (locked and unlocked wheels) there were also two groups of adult rats. These rats were given access to a locked (no exercise) or unlocked (exercise) wheel beginning at PD56. Data from one adult rat were excluded due to failure to acquire lever pressing.

Results

Two weeks of exercise in juvenile rats reduced ABA renewal of responding, but there was no change in ABA renewal in adult rats. In acquisition and extinction of lever-pressing behavior, no differences were seen between exercising and non-exercising rats (both in juveniles and adults). When tested for renewal, all four groups showed significant renewal of responding (i.e., greater responding in the non-extinction context A compared to the extinction context B), $F(1,42) = 263.96$, $p < 0.01$. However, exercising juvenile rats showed significantly less renewal than non-exercising juvenile rats, $F(1,22) = 9.44$, $p < 0.01$ (Figure 7). There was no significant difference between adult exercisers and adult non-exercisers in lever pressing in context A, $F(1, 20) = 0.54$, $p = 0.47$ (Figure 7). There was no main effect of age on distance run, or a significant age x day interaction (Figure 8).

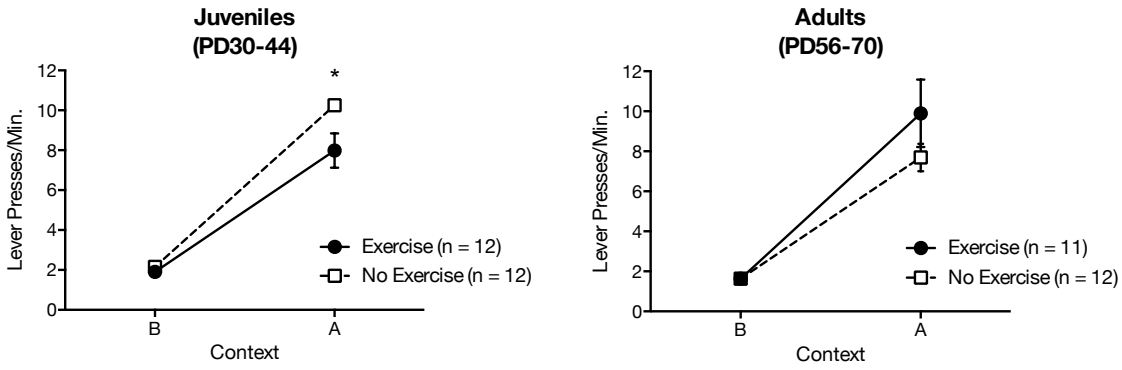

Figure 6. Lever press rates for juvenile and adult rats in renewal test. There were no differences in responding during acquisition or extinction. All groups showed renewal of responding (increased responding in context B compared to context A) while juvenile exercising rats lever pressed significantly less in the renewal context (B) than juvenile non-exercising rats. There was no difference between exercising adults and non-exercising adults.

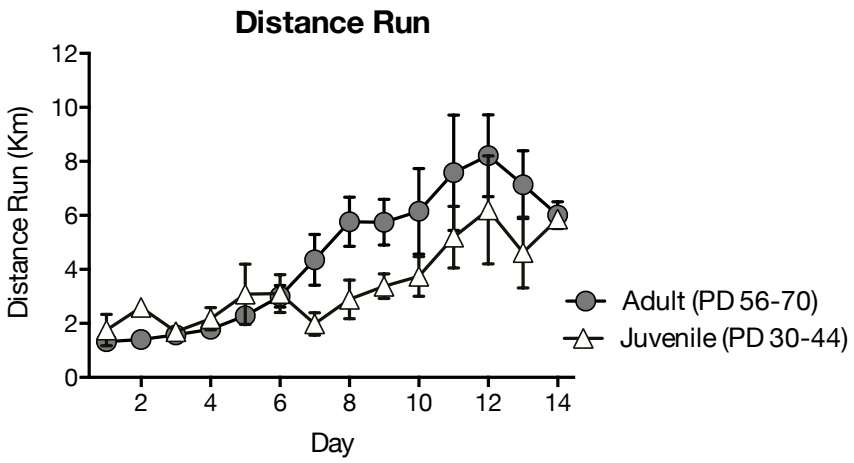

Figure 7. Average daily distance run for rats in experiment 4.

Discussion

Adult and juvenile rats were directly compared in this experiment to determine if the effects of exercise on ABA renewal are age-dependent. Renewal upon return to context A was observed across all groups. Exercising juvenile rats showed less renewal than non-exercising juveniles and exercising or non-exercising adults. This suggests that exercise reduces ABA renewal in juveniles only. Set-shifting, which is mPFC dependent (Birrell and Brown, 2000, Floresco et al., 2006) was also improved in exercising juveniles, but not adults, in our previous work. This finding lends support to the proposition that there is a critical period for development of mPFC in which it is especially susceptible to environmental influences. Additionally, it replicates the effect reported in experiment 1.

EXPERIMENTS 5 AND 6:

ROLE OF PL AND IL mPFC IN ABA RENEWAL

The changes observed in ABA renewal of extinguished lever-pressing following exercise suggest that exercise is having an effect on the mPFC, as the PL and IL regions of the mPFC have been implicated in extinction and ABA renewal of extinguished instrumental behavior (e.g., Peters et al., 2008; Willcocks & McNally, 2013). In the current literature, studies looking at the role of mPFC sub-regions in ABA renewal of extinguished operant behavior have typically used drug rewards such as cocaine, heroin, or alcoholic beer (Bossert et al., 2011; Bossert et al., 2012; Fuchs et al., 2005; Fuchs et al., 2007; Willcocks & McNally, 2013), and there is some suggestion that ABA renewal of responding for different drug reinforcers (heroin versus cocaine) relies upon different regions of the mPFC (see Bossert et al., 2011; Bossert et al., 2012; Fuchs et al., 2005; Fuchs et al., 2007). To examine the roles of the PL and IL in operant ABA renewal using the appetitive reinforcer (sucrose pellets) that we used in experiments 1, 2, and 3, experiments 5 and 6 were conducted. In these experiments, adult rats were chronically implanted with cannulae aimed at the PL (experiment 5) or IL (experiment 6) prior to the start of lever training.

Methods

Subjects. A total of 95 adult male Wistar rats (57-61 days old at delivery) obtained from Charles River Canada were used. Of this total, 4 rats were eliminated

based on an inability to locate one or both cannulas for verification, leaving a total of 91 rats (25 rats in experiment 5, 42 rats in experiment 6, 25 rats as off-site controls). Animals were housed in a temperature and humidity controlled colony room, and kept on a 12/12 hr light/dark schedule. Rats were maintained at approximately 90% of their free-feeding weight throughout the experiment.

Apparatus. The same set of operant chambers was used in these experiments as described above, with the same sucrose pellet reinforcer.

Surgery. Rats were anesthetized with isoflurane and stereotaxic surgery was performed in order to bilaterally implant guide cannulae (26 gauge, Plastics One) in the PL (experiment 5) or IL (experiment 6) mPFC. Coordinates used were +3.0 mm from bregma, ± 0.75 mm from midline, and -3.0 mm ventral from the skull (PL) and +3.0 mm from bregma, ± 2.66 mm from midline, and -4.71 mm ventral at a 24° angle (IL), and +3.0 mm from bregma, ± 2.66 mm from midline, and -6.10 mm ventral at a 24° angle (ventral off-site) (coordinates adapted from Willcocks & McNally, 2013). Following surgery, rats were given 5-6 days of recovery.

Instrumental Conditioning and Extinction. The two experiments investigating PL and IL were run separately (during different calendar months), but used the same behavioral methods. Conditioning and extinction methods used in these experiments were identical to those used in previous ABA experiments.

Baclofen/Muscimol Infusions and Testing. Testing took place the day after the final extinction session. Rats were given an infusion of 0.9% saline vehicle (control) or baclofen/muscimol (B/M) (1.0mM/0.1mM; Sigma Aldrich, St Louis, MO) dissolved in

0.9% saline to temporarily inactivate the targeted region (PL in experiment 5; IL in experiment 6). Internal cannulae (33 gauge, Plastics One) were inserted bilaterally into guide cannulae. Internal cannula tips protruded 1 mm below the guide cannula tip. An infusion of 0.5 µL per side was delivered at a rate of 0.25 µL per minute using a microinfusion pump. Following completion of the infusion, the internal cannulae were left in place for 1 minute to allow diffusion of the drug or saline away from the cannula tips. They were then removed, dummy cannulae replaced, and the rat was placed in the transportation container. Time between the end of infusion and the start of testing ranged from approximately 30-45 minutes.

All animals were tested in a within-subject manner (Bouton et al., 2011). Each rat was tested in two separate 10-minute sessions, one in context A and one in context B (test order counterbalanced). As in extinction sessions, lever presses did not result in a sucrose pellet. The two test sessions were separated by approximately 30 minutes.

Infusion Spread Visualization/Histology. In order to determine the extent of the spread of the B/M infusion, 0.5 µL of 0.5 mg/mL fluorophore-conjugated muscimol (FCM) in 0.9% saline (muscimol, BODIPY®TMR-X conjugate, Molecular Probes) was infused (see Allen, Narayanan, Kholodar-Smith, Zhao, Laubach, & Brown, 2008) in a subset of 4 rats (PL infusion = 2; IL infusion = 2) the day after testing using the same infusion procedure as above. Rats receiving FCM infusions were sacrificed approximately 30 minutes following infusion, to mimic time of testing after drug infusion, and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and sectioned at 50µm on a cryostat. FCM was

visualized using a Zeiss LSM 7 multiphoton microscope. Images were collected at 10x and a tile scan was used in order to visualize a large field of view (i.e., an entire hemisphere of a coronal section). Fluorescent images were overlaid on brightfield images of cresyl violet stained sections.

All other rats were transcardially perfused with 0.9% saline followed by 10% formalin. An electrolytic lesion was made in order to visualize the cannula tip in rats that did not receive FCM. Brains were cryoprotected in 30% sucrose and sectioned at 50μm on a cryostat. Sections used for cannula verification were stained with cresyl violet. Three independent, blind observers performed cannula verification by marking the location of the electrolytic lesion on corresponding diagrams from a rat brain atlas (Paxinos & Watson, 2007). Data from animals where the location of one or both cannulae tip(s) could not be identified were excluded from analyses. The final number of animals excluded from the study was 4.

Results

Experiment 5: PL mPFC inactivation. There were no differences between groups in acquisition (vehicle group mean ± SEM = 16.5 ± 2.0 lever presses per minute in the final session; B/M group mean ± SEM = 19.7 ± 1.5 lever presses per minute in the final session) or extinction (vehicle group mean ± SEM = 6.3 ± 0.6 lever presses per minute in first session and 1.2 ± 0.2 lever presses per minute in the final session; B/M group mean ± SEM = 7.1 ± 0.9 lever presses per minute in the first session and 1.0 ± 0.2 lever presses per minute in the final session), as expected, since no infusions were made during these sessions (all p's > 0.05). During testing, renewal was observed in both groups but PL

inactivation reduced responding in context A (renewal context) compared to controls. A 2 (B/M, vehicle) X 2 (lever presses A, lever presses B) mixed model ANOVA revealed a significant main effect of context ($F(1,23) = 231.21$, $p < 0.001$) and a significant context by infusion type interaction ($F(1,23) = 12.24$, $p = 0.002$). Follow-up tests of the interaction revealed significantly less responding in the PL inactivation group in context A (renewal), ($F(1,23) = 9.25$, $p = 0.006$), but not in context B (extinction context), ($F(1,23) = 0.02$, $p = 0.88$), compared to vehicle controls (Figure 9A).

Experiment 6: IL mPFC Inactivation. Again, as expected, there were no differences between groups in acquisition (vehicle group mean ± SEM = 17.5 ± 3.1 lever presses per minute in the final session; B/M group mean ± SEM = 15.7 ± 1.9 lever presses per minute in the final session) or extinction (vehicle group mean ± SEM = 7.0 ± 0.6 lever presses per minute in the first session and 1.0 ± 0.1 lever presses per minute in the final session; B/M group mean ± SEM = 6.3 ± 0.7 lever presses per minute in the first session and 1.4 ± 0.3 lever presses per minute in the final session) (all p's > 0.05). During testing, renewal was observed in the vehicle control group but not in the IL inactivation group; IL inactivation reduced responding in context A (renewal context) and increased responding in context B (extinction context) compared to controls. A 2 (B/M, vehicle) X 2 (lever presses A, lever presses B) mixed model ANOVA revealed a significant main effect of context ($F(1,40) = 27.69$, $p < 0.001$) and a significant context by infusion type interaction ($F(1,40) = 13.10$, $p = 0.001$). Follow-up tests of the interaction effect revealed significantly less responding in the IL inactivation group in context A (renewal context), ($F(1,40) = 9.15$, $p = 0.008$), and significantly more responding in the IL inactivation

group in context B (extinction context), ($F(1,40) = 7.67$, $p = 0.004$), compared to vehicle controls (Figure 9B).

Off-site (control) infusions. For rats with cannulae implanted ventral or lateral to the IL, there were no differences between groups in acquisition (vehicle group mean ± SEM = 19.1 ± 2.6 lever presses per minute in the final session; B/M group mean ± SEM = 19.7 ± 0.5 lever presses per minute in the final session) or extinction (vehicle group mean ± SEM = 6.5 ± 0.8 lever presses per minute in the first session and 1.5 ± 0.2 lever presses per minute in the final session; B/M group mean ± SEM = 7.0 ± 0.3 lever presses per minute in the first session and 1.3 ± 0.1 lever presses per minute in the final session) (all p's > 0.05). Both groups showed renewal, but infusion type had no effect on responding. A 2 (B/M, vehicle) X 2 (lever presses A, lever presses B) mixed model ANOVA revealed a main effect of context ($F(1,22) = 52.31$, $p < 0.001$), but no significant interaction of context and infusion type ($F(1,22) = 0.03$, $p = 0.87$) (Figure 9C).

Histology. Example images of histological sections taken following test are shown in Figure 10 for FCM infusions made in PL and IL. FCM images are overlaid on one half of the cresyl violet stained sections. FCM infusion indicated that spread was minimal, and likely did not extend beyond the targeted region. Figure 11 depicts cannula tip placements for all rats used in these experiments.

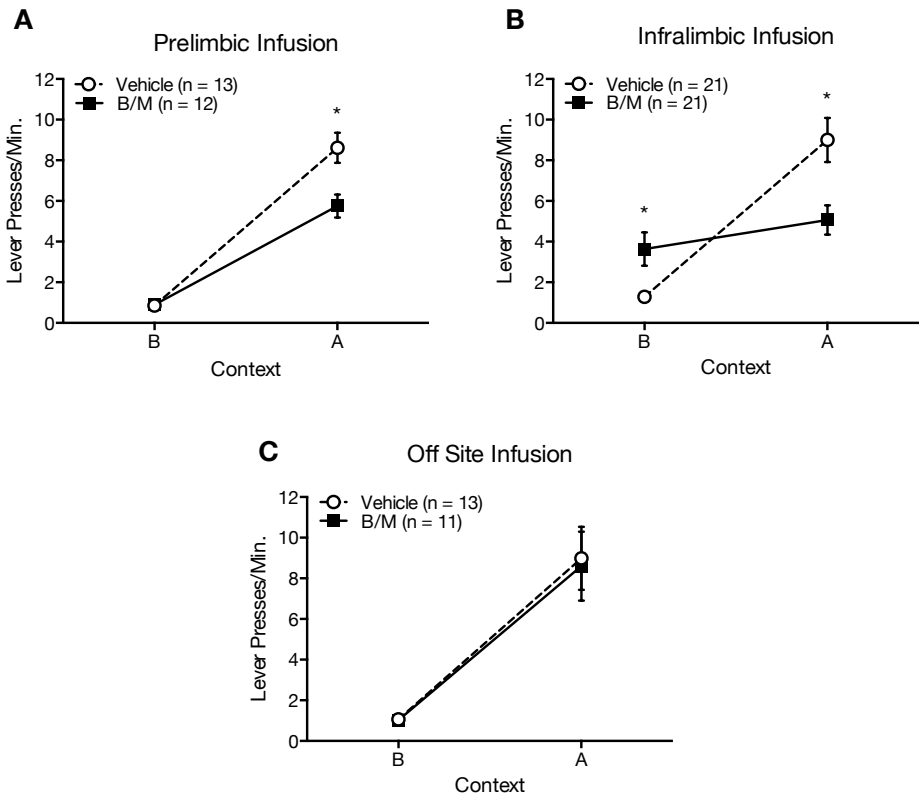

Figure 8. Lever press rates for rats with infusions in PL (A), IL (B), or off-site control (C). Rats receiving B/M in PL showed less renewal than vehicle animals. Rats receiving B/M in IL responded more than vehicle animals in context B, while responding less than vehicle rats in context A. There was no difference between vehicle and B/M rats infused at an off-site mPFC target (C).

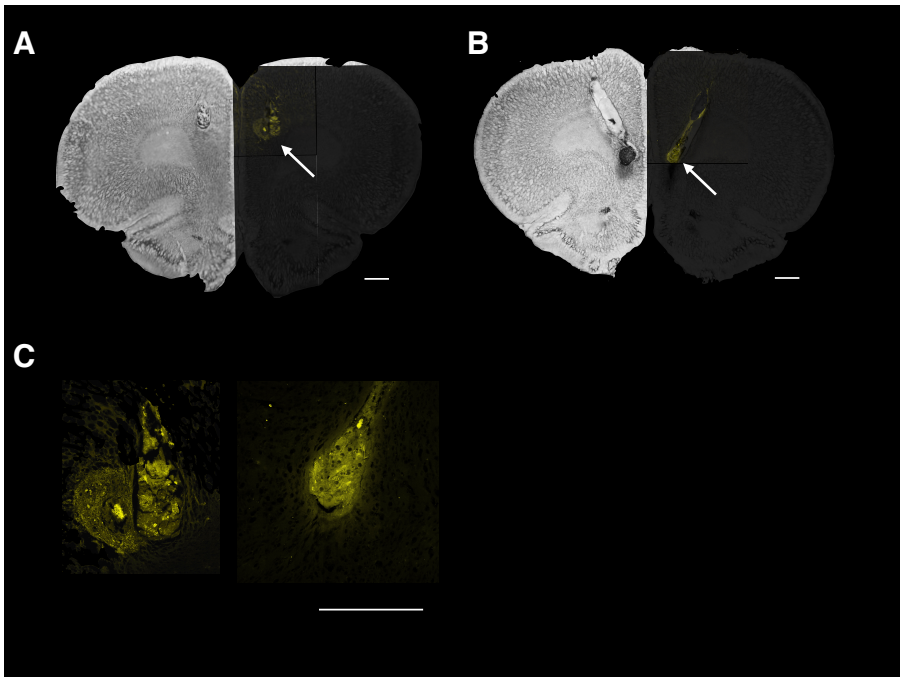

Figure 9. Fluorophore-conjugated muscimol (right side of section, panels A and B) overlaid on cresyl violet stained mPFC sections showing (A) PL and (B) IL cannula tip location. Infusion spread detail from PL (left) and IL (right) sections (C). Scale bar = 1mm.

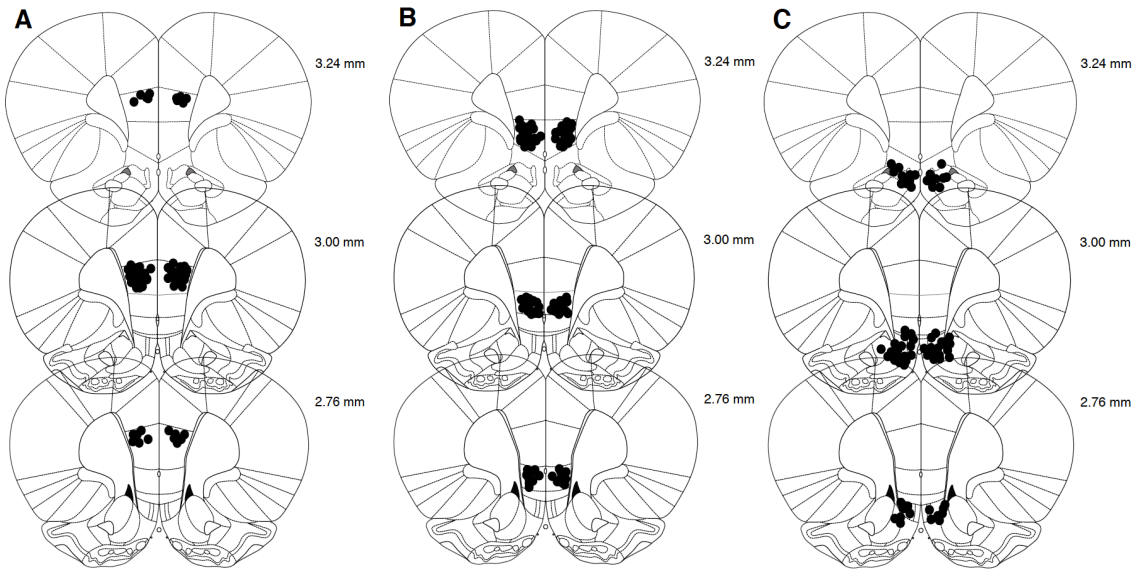

Figure 10. Cannlua tip placements for PL (A), IL (B), and off-site controls (C).

Discussion

Experiments 5 and 6 examined the role of the PL and the IL mPFC in renewal following extinction of instrumental behavior with a sucrose reward. Renewal in context A was reduced by inactivation of the PL or IL. Conversely, although PL inactivation had no impact on responding in context B (extinction context), inactivation of the IL increased responding in this context.

Several controls were used in these studies to improve interpretation of our results. Using FCM, we were able to demonstrate very minimal spread of our experimental drug infusion. It is important to note that the spread of FCM from a 0.5 μL dorsal mPFC infusion in a study by Allen et al. (2008) was 0.5-1.0 mm, which is quite comparable to what we observed here. In any infusion study, spread of the drug beyond the target region is a concern—particularly when regions of interest are adjacent to one another. Unintended spread of B/M could easily affect both the PL and IL due to their close anatomical proximity, though our FCM infusions suggest minimal spread to surrounding regions.

One possible criticism is that the FCM infusion underrepresents the actual spread of B/M due to the size of the fluorescent tag (the molecular weight of FCM is 607.46 g/mol; the molecular weight of muscimol hydrobromide is 195.01 g/mol; the molecular weight of baclofen hydrochloride is 250.12 g/mol). While the effects of FCM versus B/M were not compared in our study, Allen et al. (2008) showed that the behavioral effects of FCM infusion were similar to those of a muscimol (114.10 g/mol) infusion despite their different molecular weights. Second, we included a control group of animals

that received B/M infusions in a mPFC location outside of the PL or IL. This allowed us to demonstrate that our observed results are not due to general effects of inactivation on the mPFC, but are specific to the PL or IL. Finally, small diameter cannulae (26 gauge) aimed at the IL were implanted at a steep angle (24°) in order to minimize incidental damage to the PL, which lies directly dorsal to the IL.

The reduction in ABA renewal in rats with an inactivated PL suggests that while this region plays some role in ABA renewal, it is not the sole substrate underlying this phenomenon. One possible role of the PL is retrieval of the original learning that occurred during acquisition. This region may promote expression of the association between the context and reward availability. PL inactivation could have reduced control by a context-response association in the relatively simple situation studied here. Other evidence suggests that lesioning or inactivation of the PL prior to instrumental conditioning can discourage expression of response-outcome learning (Balleine & Dickinson, 1998; Corbit & Balleine, 2003; Coutureau, Marchand, & Di Scala, 2009; Killcross & Coutureau, 2003; Ostlund & Balleine, 2005; Tran-Tu-Yen, Marchand, Pape, Di Scala, & Coutureau, 2009). Although the present data suggest that PL inactivation reduces excitatory control of the response that occurs when the animal is returned to the conditioning context after extinction, they are consistent with the PL influencing the expression of either an excitatory context-response association or a hierarchical context-(response-outcome) relation.

Inactivation of the IL increased responding in the extinction context and decreased responding in the renewal context relative to control animals. Again, because

we know that both the B/M and saline vehicle groups learned equivalently during acquisition and extinction, the increased responding in the extinction context may indicate that without the IL, these animals are impaired at expressing an inhibitory context-response association. If we assume that the extinction context forms an inhibitory association with the response (Todd, 2013; Todd et al., 2014), then inactivation of the IL may impair the expression of this inhibitory context-response association, resulting in more responding in the context in which lever pressing was extinguished. The additional reduction of responding in the acquisition context (i.e., reduction in renewal) with inactivation of the IL may be due to impairment in expression of an excitatory context-response association or a context-(response-outcome) relation, as with the PL. IL lesioning or inactivation before or after instrumental conditioning can discourage expression of stimulus-response learning (Coutureau & Killcross, 2003; Killcross & Coutureau, 2003). These experiments add to the somewhat unclear literature on the roles of the PL and IL in renewal, providing evidence for their contributions to ABA renewal of responding to a non-drug reinforcer.

Together with the data showing that exercise, *when it takes place during development*, reduces ABA renewal, these experiments provide insight into what brain regions may be underlying this exercise-induced behavioral change. Of note, the reduction in responding in the renewal test in rats infused with B/M is numerically almost identical to the reduction seen following exercise. Exercise may be preferentially affecting the PL or, synaptic pruning occurring in developing rats may shift the balance of excitatory and inhibitory inputs to the mPFC. If there is synaptic pruning as a result of

exercise, specifically during exercise, this may result in a more optimal balance of inputs to the mPFC or PL in particular, resulting in improved inhibitory learning.

EXPERIMENT 7: mPFC DENDRITIC COMPLEXITY

To explore the possibility that exercise results in morphological changes in the mPFC which may underlie the behavioral changes we observed, we compared neuronal complexity in adult and juvenile rats that had a locked or unlocked running wheel for two weeks. Altering the number of dendritic spines can have an effect on excitatory inputs to pyramidal neurons in the PFC. Development is a time period in which dendritic pruning and remodeling is at a high point (Gould et al., 1991, Funahashi, 2001, Kim et al., 2009, Gamo and Arnsten, 2011). With this in mind, I examined pyramidal neurons in the mPFC of young or adult rats that exercised and compared them to rats that had not exercised. I predicted that developing rats that ran for two weeks would have more dendritic spines than non-exercising rats of the same age. Additionally, considering the lack of effect on the PFC-dependent behaviors described above, I predicted that little or no change in dendritic complexity would be observed in adult exercising rats. Indeed, there are some data to support the possibility of age and experience affecting changes in PFC dendrites (Bloss et al., 2011). Here I asked whether exercise would induce this type of plasticity, and if it is age dependent or ubiquitous across the lifespan.

Methods

Male Wistar rats obtained from Charles River Canada were used, as described above. Adults were given access to a locked or unlocked running wheel starting at PD56 and juveniles at PD30. Animals were not food restricted. After 14 days of wheel access,

all rats were sacrificed by an overdose of sodium pentobarbital and transcardially perfused with cold 0.9% saline.

Golgi-Cox solution consisted of 5% potassium dichromate and 5% mercuric chloride mixed 1:1. 5% potassium chromate was then diluted 4:10 with the potassium dichromate/mercuric chloride solution. The solution was stored in the dark for 5 days and then filtered. Perfused brains were placed whole into Golgi-Cox solution and stored for 12-15 days in the dark at room temperature. Solution was changed every other day for the first 4 days. Whole brains were then transferred to a 30% sucrose solution for cryoprotection for approximately 48 hours. Brains were then blocked, flash frozen using isopentane and dry ice, and then mounted onto a cryostat chuck using distilled water. Sections were taken at -30°C at a thickness of 150μm and mounted directly onto slides. The following day, tissue was processed through a series of distilled water washes, followed by ammonium hydroxide, Kodak D19 developer and Ilford rapid fix (diluted 1:4 with distilled water). After several distilled water washes, the tissue was then dehydrated through an increasing series of ethanol (50%, 70%, 90%, 100%), cleared in histoclear, and coverslipped.

Tracings were done at 600x magnification using Neurolucida (MBF Technologies, Williston, VT). Selection criteria were based on methods used in previous work examining Golgi-Cox stained PFC neurons (Wellman, 2001). Layer II/III pyramidal cells were identified as having a single apical dendritic tree, two or more basilar dendritic trees, and with no broken/incomplete branches, or obstruction by surrounding neurons. Of the neurons that met criteria, 4-6 were randomly chosen (half left hemisphere, half

right; split between dorsal and ventral mPFC). Average dendritic length, branch number, and spine number was included from 8 rats per group, with each rat's average coming from 4-6 traced neurons. Data were analyzed using a 2 (adult, juvenile) x 2 (exercise, no exercise) ANOVA.

Results

Number of dendritic spines, number of dendritic branches, and length of branches were analyzed. For dendritic spines, there was a main effect of exercise ($F(1,28) = 4.19$, $p = 0.05$), but not age ($F(1,28) = 0.03$, $p = 0.86$). This was qualified by a significant interaction between age and exercise ($F(1,28) = 7.64$, $p = 0.01$) (Figure 12A). Number of branches was not affected by exercise ($F(1,28) = 0.09$, $p = 0.77$) or age, $F(1,28) = 0.59$, $p = 0.45$ (Figure 12B). Branch length was not affected by exercise ($F(1,28) = 1.77$, $p = 0.20$), but was by age ($F(1, 28) = 10.05$, $p < 0.01$). There was a significant exercise x age interaction ($F(1,28) = 4.20$, $p = 0.05$) (Figure 12C). No differences were revealed between neurons in the dorsal region of the mPFC and neurons in the ventral region. A photomicrograph of pyramidal cells in juvenile exercising and juvenile non-exercising rats is shown in Figure 13. There were no significant differences in distance run between adults and juveniles (Figure 14).

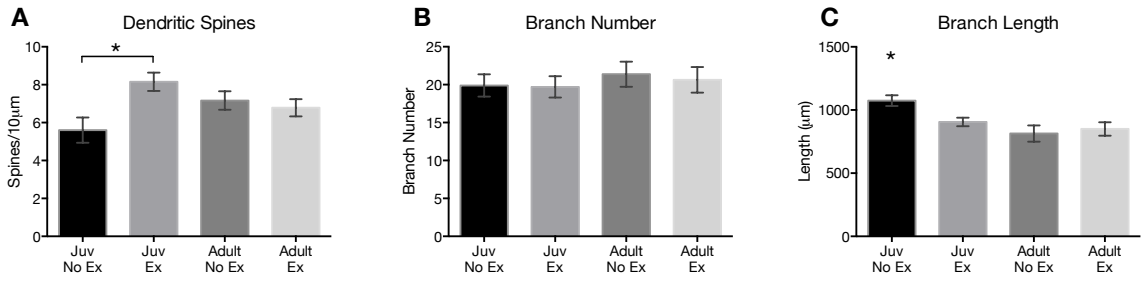

Figure 11. Number of dendritic spines (A), dendritic branches (B), and length of dendritic branches (C) in exercising and non-exercising adult and juvenile rats. Exercising juvenile rats had significantly more dendritic spines than juveniles that did not exercise. Non-exercising juveniles had significantly longer dendritic branches than juvenile exercisers, and both exercise and non-exercising adults.

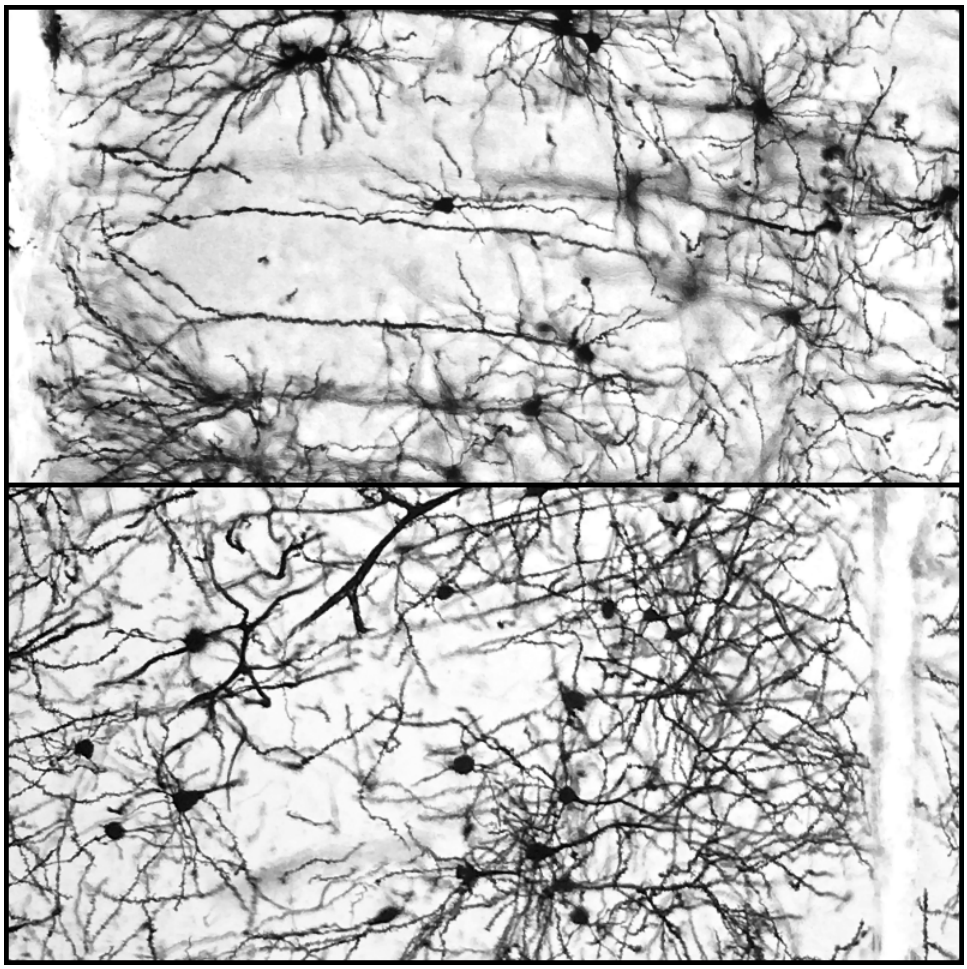

Figure 12. Golgi-Cox stained neurons in mPFC of juvenile rat that exercised for two weeks (top panel) and a juvenile rat that did not exercise (bottom panel).

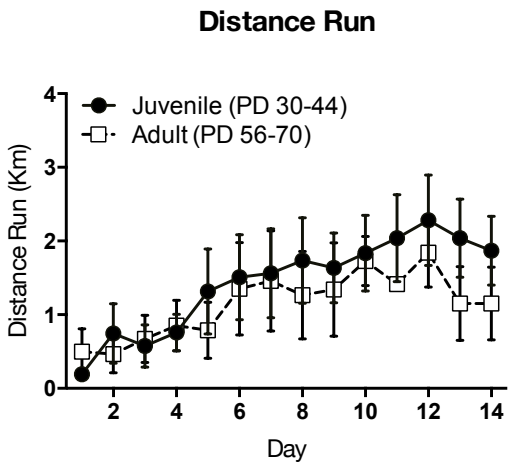

Figure 13. Average daily distance run by juvenile and adult rats included in experiment 7.

Discussion

Using Golgi-Cox staining to visualize pyramidal neurons in the mPFC, this experiment showed that there were several morphological differences between juveniles and adults, and between rats that had exercised or not. As predicted, juvenile rats that exercised had more dendritic spines than juveniles that had not exercised. Exercising juvenile rats shorter average dendritic branch length, while there was no difference between juveniles in the number of dendritic branches. In adults, exercise had no effect on any measure of dendritic complexity. These results show that exercise only had an effect on the mPFC neurons of juvenile rats, which may suggest that exercise is only effective in modulating the morphology of these cells while the mPFC is still developing, which is similar to results from previous studies (Bloss et al., 2011, Selemon, 2013). Additionally, the dendritic branches of exercising juveniles were shorter than non-exercising juveniles, perhaps indicating that more synaptic pruning had occurred in these

animals. In adults, the process of synaptic pruning may already be complete, or nearly so, leaving little room for exercise to have an impact on neurons in this way. Overall, these results fit with the idea that exercise may be particularly important during development, when the mPFC in particular is receptive to plasticity.

EXPERIMENT 8: EXERCISE AND mPFC NET EXPRESSION

To further explore the neurobiological underpinnings of the behavioral changes seen following exercise, this experiment compared NET protein expression in non-exercising and exercising rats at two ages, adulthood and adolescence. Several commonly prescribed drugs to treat prefrontal-related pathologies, such as ADHD, target NET in order to increase noradrenergic neurotransmission (Vanicek et al., 2014, Somkuwar et al., 2015). Prefrontal NE has been shown to play an important role in various types of attentional tasks, including set-shifting (McGaughy et al., 2008). Additionally, there is evidence to suggest that exercise has modulatory effects on NET levels in the PFC (Robinson et al., 2015). The prefrontal NE system has been implicated in many of the behaviors we have examined, such as set-shifting, as well as other "higher-order" cognitive abilities (Devauges and Sara, 1990, Tronel et al., 2004, Ramos and Arnsten, 2007, Caetano et al., 2012). In this experiment, mPFC tissue from rats used in experiment 4 was analyzed for NET protein levels. I predicted that exercise would decrease NET in mPFC, with the assumption that less NET expression would indicate more synaptically available NE. An exercise-induced reduction of NET may underlie the behavioral improvements observed in the mPFC-dependent tasks described above.

Methods

The day following testing (experiment 4) rats were sacrificed with an overdose of sodium pentobarbital and the brain rapidly removed, mPFC dissected out, and flash frozen on dry ice. Tissue collection occurred within 2 hours of the time testing had

occurred the previous day to minimize effects of diurnal rhythms in NE levels. Each group had samples from 12 rats. Three separate assays were run, each with 4 samples/group. Data from the three runs were normalized within run to the no exercise control.

$$adult\ rat\ normalized\ data = \frac{NET\ for\ individual\ rat}{mean\ NET\ adult/no\ exercise}$$

$$juvenile\ normalized\ data = \frac{NET\ for\ individual\ rat}{mean\ NET\ juvenile/no\ exercise}$$

mPFC tissue was bead-homogenized in homogenization buffer (10mM Tris-HCl, pH 7.6, 1 mM EDTA, 200 mM sucrose, HALT protease & phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA) using a FastPrep-24 (MP Biomedicals, Santa Ana, CA) for 30 sec at 6.5 m/s. The resulting homogenates were centrifuged for 2 min at 1000 x g. Supernatants were centrifuged for 90 min at 16,100 x g. Protein pellets were solubilized by sonication in Tris buffer (10mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% Triton X-100, HALT protease & phosphatase inhibitor cocktail). Total protein concentrations were determined using the Coomassie Plus reagent (Pierce Biotechnology, Rockford, IL). Protein samples (33 g) were resolved on 12% LDS-PAGE gels (Life Technologies, Carlsbad, CA) then transferred to Immobilon-FL PVDF membranes (Millipore, Billerica, MA). Membranes were blocked and incubated with primary antibodies for NET (Alpha Diagnostic International, Inc., San Antonio, TX) and sodium/potassium ATPase 1 (Cell Signaling Technology, Beverly, MA). Quantitative

analyses were performed by measuring the relative fluorescent unit (RFU) ratio of NET immunoreactive bands normalized to sodium potassium ATPase immunoreactive bands in the same lane for each sample. RFU measurements were also normalized by region of interest (ROI) area. Analyses were carried out using a Licor Odyssey imager and application software version 3.0.30 (Licor Biosciences, Lincoln, NE). RFU measurements were taken from the band at 80 kDa, which indicates mature NET (Melikian et al., 1994)

Results

A 2 (age) x 2 (exercise) ANOVA revealed a borderline significant main effect of age ($F(1, 44) = 3.79$, $p = 0.058$) and age x exercise interaction ($F(1,44) = 3.80$, $p = 0.058$). Exercising adults had significantly more NET expression than exercising juveniles ($F(1,22) = 6.47$, $p = 0.02$) (Figure 15). An image of one of three blots included in these analyses is shown in Figure 16.

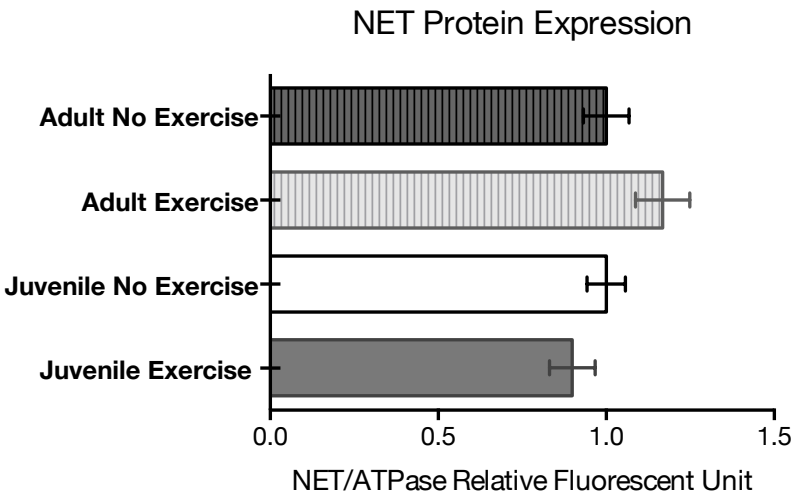

Figure 14. mPFC NET protein expression at 80 kDa band normalized over 3 runs (each run n = 4/group).

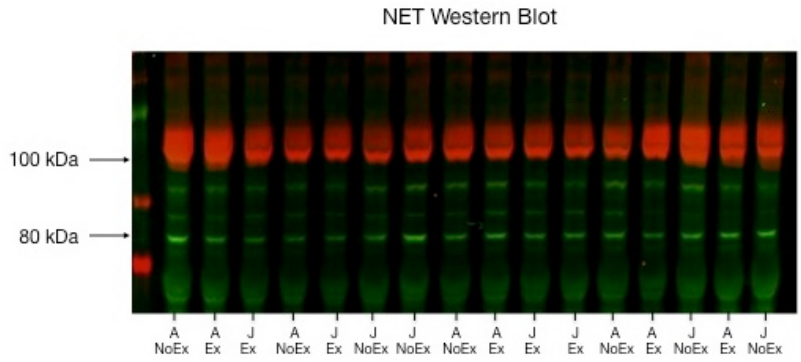

Figure 15. Raw image of one of three runs looking at NET protein expression in mPFC tissue from adult and juvenile rats in exercise or non-exercise groups. In this run each group $n = 4$. NET is visualized in green and Na+/K ATPase (loading control) in red.

Discussion

Two weeks of exercise during development resulted in less NET expression compared to adults that exercised, however, there was no difference between juveniles that exercised or did not exercise. The reduction of NET in exercising juveniles compared to exercising adults may suggest that exercise effects on NET expression are specific to development. Additionally, the normalized data suggest that in contrast to the exercise-induced reduction of NET in juveniles, there was an exercise-induced increase in adults. Again, this is suggestive of the specificity of the effects exercise has on the mPFC depending on developmental time point. The data were also normalized to the expression levels in adult no-exercise rats, since this group may represent the most "normal" expression of NET (since the other three groups were either able to run, were juveniles, or both). Analysis of the data normalized this way revealed a nearly identical pattern to

results reported above. Therefore, it appears that changes in NET expression following exercise depend on the age/developmental stage of the animal.

GENERAL DISCUSSION

The experiments presented here add to the current literature on exercise, development, the mPFC, and renewal of instrumental conditioning. Here it has been demonstrated that physical exercise can reduce renewal of responding when a return to the acquisition context occurs, but only if the exercise takes place while the animal is developing. Building upon this result, I have shown that the sub-regions of the mPFC play dissociable roles in ABA renewal of instrumental behavior when a food reinforcer is used. Finally, to establish that these behavioral effects of exercise are underpinned by morphological changes, I have shown here that exercise during development affects complexity of neurons in the mPFC.

The finding that exercise reduced ABA renewal in juvenile rats has several possible implications. The reduction in renewal in exercising rats may indicate that they are more successful at inhibiting non-productive behaviors than non-exercising rats. This possibility is supported when we look at the lever-pressing rats during test divided into 30-second bins. While exercising juvenile rats do still show ABA renewal (that is, increased lever pressing in context A compared to context B), their rate of responding drops off quickly, while non-exercising rats take longer to suppress lever pressing. Another possible explanation of the reduction of renewal could be better consolidation of the information learned during extinction. However, in experiment 2, where an AAB paradigm was used, the outcome does not support this idea. When acquisition and extinction took place in the same context (A), exercisers still showed less renewal when

tested in a new context (B) than non-exercisers. Indeed, it is impossible to say if this was a true reduction of AAB renewal or a lack of renewal due to a possible floor effect in response rates in exercising rats. However, given the fact that AAB is numerically a less robust form of renewal than ABA, it seems plausible to conclude that exercising rats were again suppressing or inhibiting their behavior in the test context (B) more effectively than non-exercising rats.

To continue to explore the neurobiology underlying the changes in ABA renewal following exercise, I manipulated the PL and IL mPFC to determine the importance of these areas in our particular paradigm. The results from PL or IL inactivation prior to ABA renewal test are the first to indicate specific roles of mPFC subregions in expression of extinction and ABA renewal of instrumental responding in a food reinforcement preparation. In contrast to our experiments using a food reinforcer, the roles of the PL and the IL in extinction and ABA renewal of drug-seeking may depend upon which drug serves as the reinforcer (Bossert et al., 2013; Peters et al., 2013). For example, Bossert et al. (2011) found that ABA renewal of lever-pressing for heroin was decreased following inactivation of the IL while inactivation of the PL had no effect. In contrast, Willcocks and McNally (2013), examining nose-poking for alcoholic beer, and Fuchs et al. (2005), examining lever-pressing for cocaine, found no effect of IL inactivation on ABA renewal, instead demonstrating that PL inactivation reduced ABA renewal. None of those studies reported an effect of PL or IL inactivation on expression of extinction, although Peters et al. (2008) found that inactivation of the IL after extended extinction training reduced expression of extinction.

The behavioral mechanisms of the extinction and renewal of food-reinforced behavior have been studied more intensively (e.g., Bouton & Todd, 2014). One possible role of the PL is in expression of the original learning that occurred during acquisition in context A. Because all rats underwent acquisition and extinction before receiving any infusion, and all animals acquired and extinguished responding to the same level, we can conclude that this relative reduction of responding is not due to a failure to learn or consolidate information about the context, the response, or the reward. Bouton and colleagues have provided evidence that, unlike a context switch after Pavlovian conditioning, which usually does not affect the expression of the conditioned response to a conditioned stimulus, a context switch after instrumental conditioning causes a decrement in the expression of the instrumental response (Bouton et al., 2011; Bouton, Todd, & Leon, 2014; Thrailkill & Bouton, 2015; Todd, 2013; see Bouton & Todd, 2014, for a review). Such a result could occur if the conditioning context had entered into a direct association with the instrumental response (Thrailkill & Bouton, 2015) or if the context had come to signal the relationship between the response and the reinforcing outcome in a more "hierarchical" manner (Trask & Bouton, 2014; see Bouton & Todd, 2014, for more discussion). In principle, either of these processes could have been affected by inactivation of the PL here. It is worth noting that the ability for a context to signal the response-reinforcer relationship (Trask & Bouton, 2014) may depend on complex discrimination training with several response-outcome relations; when animals are given simple acquisition training with a single response-outcome relation in one

context, the response decrement that occurs with a context switch appears to result from the loss of the direct context-response association (Thrailkill & Bouton, 2015).

One implication might be that PL inactivation could have reduced control by a context-response association in the relatively simple situation studied here. Other evidence suggests that lesioning or inactivation of the PL prior to instrumental conditioning can discourage expression of response-outcome learning (Balleine & Dickinson, 1998; Corbit & Balleine, 2003; Coutureau, Marchand, & Di Scala, 2009; Killcross & Coutureau, 2003; Ostlund & Balleine, 2005; Tran-Tu-Yen, Marchand, Pape, Di Scala, & Coutureau, 2009). Although the present data suggest that PL inactivation reduces excitatory control of the response that occurs when the animal is returned to the conditioning context after extinction, they are consistent with the PL influencing the expression of either an excitatory context-response association or a hierarchical context (response-outcome) relation.

Inactivation of the IL increased responding in the extinction context and decreased responding in the renewal context relative to control animals. Again, because we know that both the B/M and saline vehicle groups learned equivalently during acquisition and extinction, the increased responding in the extinction context may indicate that without the IL, these animals are impaired at expressing an inhibitory context-response association. If we assume that the extinction context forms an inhibitory association with the response (Todd, 2013; Todd et al., 2014), then inactivation of the IL may impair the expression of this inhibitory context-response association, resulting in more responding in the context in which lever pressing was extinguished. The additional

reduction of responding in the acquisition context (i.e., reduction in renewal) with inactivation of the IL may be due to impairment in expression of an excitatory context-response association, as with the PL. IL lesioning or inactivation before or after instrumental conditioning can discourage expression of stimulus-response learning (Coutureau & Killcross, 2003; Killcross & Coutureau, 2003).

There is some suggestion that the processes underlying renewal of instrumental responding are not identical in food-reinforced and drug-reinforced paradigms. ABA, AAB, and ABC renewal have all been convincingly demonstrated using food reinforcers, while only ABA has been shown in drug reinforced paradigms (Todd et al., 2014a). The data presented here suggest that the PL does indeed play a role in renewal of extinguished lever pressing for sucrose, but is not the sole substrate underlying this behavior.

Understanding the neural underpinnings of extinction and renewal, and what factors may modulate them, has important translational value. An inability to inhibit unwanted behaviors underlies many neuropsychiatric diseases, including ADHD, PTSD, problem gambling, overeating, and drug addiction (Anderson et al., 2001, Gardner and Steinberg, 2005, Hauser et al., 2014). These disorders are also associated with sub-optimal PFC function, and tend to manifest around the time of puberty (Anderson et al., 2001, Gardner and Steinberg, 2005, Hauser et al., 2014). Considering this, research examining the PFC and its functions, particularly when it may be most vulnerable to environmental influence, is critical for not only the basic understanding of how these mechanisms work, but also for clinical application.

The data presented here suggest that exercise, when it occurs early in life, can reduce renewal of instrumental responding. Coupled with this, it is also demonstrated that only rats that had a running wheel during development had increased numbers of dendritic spines. An increase in spine density may indicate more excitatory inputs to the mPFC. Morphological changes may also contribute to a "fine-tuning" of this region across development, which appears to be aided by physical exercise. These underlying neurobiological changes could play a role in the behavioral changes we observed by improving overall PFC function, or perhaps altering the balance of excitatory and inhibitory inputs in a way that allows for optimal learning and expression of behaviors involving inhibition.

Adult rats that had exercised showed no differences from non-exercising adults in mPFC neuron morphology. These findings suggest that there is a critical window for mPFC plasticity (at least plasticity from physical exercise), and that this plasticity may have an important impact on behavior. The lack of changes seen in mPFC neurons in adults may partly explain why we saw no change in renewal after exercise. It may also be one reason no improvement in set-shifting, another PFC-dependent behavior, was seen in adults.

In the literature there is evidence for sexually dimorphic changes in mPFC neurons during and after puberty. Around the onset of puberty, female, but not male rats, lose a significant number of neurons in the mPFC (Willing and Juraska, 2015). Number of neurons and white matter volume in adulthood were shown to depend on circulating estrogen during puberty (with estrogen being associated with fewer cells and less white

matter volume) while testosterone presence or absence during development had no effect (Koss et al., 2015). This is an important consideration for future research. To expand and further elucidate changes in the mPFC during development and how they are manifested behaviorally, studies that directly compare males and females are essential.

Expanding on the results discussed here is important for several reasons, as discussed above. One key aspect that should be studied is the longevity of the effects of exercise on instrumental learning. We tested rats during their access to a running wheel. Whether or not these effects would persist if exercise were stopped is an aspect not explored by these experiments. Along those lines, giving longer than 14 days of wheel access prior to acquisition or extinction could potentially lead to different results. Perhaps 14 days is the minimum amount of physical exercise required to exhibit the behavior changes we observed. Though unlikely, differences in acquisition rate or extinction may have been seen if rats had had more running experience before that stage of our instrumental conditioning procedure. Exercise effects may be more persistent in juveniles than in adults. For example, Hopkins and Bucci found that juvenile rats that exercised for four weeks could better discriminate in a novel object recognition task and had increased BDNF in the perirhinal cortex. Both of these effects remained when tested four weeks later. However, in adult rats, the behavioral improvement was only observed immediately after exercise had taken place—no benefit was seen two weeks later (Hopkins et al., 2011).

Taken together, the data presented here contribute several important findings to the literature on exercise, behavior, and development. I demonstrated that running wheel

exercise reduced ABA and AAB renewal in juvenile rats, adding to our previous data that juveniles exhibit improvements in mPFC-mediated behavior. By inactivating the PL or IL mPFC prior to a test of ABA renewal, I disambiguated the roles these mPFC regions play in renewal for a food reinforcer. Finally, morphological changes in the mPFC due to exercise were shown to only occur in young rats. The finding that young rats that had exercised during development had more dendritic spines and shorter dendritic branches than non-exercising cohorts again suggests that this brain region may be primed for plasticity at this developmental time point. Exercise does not appear to affect neuron morphology when exercise is during adulthood. Together these data suggest that there is a critical period of development during which the mPFC in particular may be especially vulnerable to environmental influences, positive or negative.

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